

REMARKS

Claims 1, 4, 5, 7, 10-16 and 23 are pending in this application. Claims 17-20 were withdrawn from consideration as being drawn to non-elected Groups and subsequently cancelled. By this Amendment, claim 1 is amended to recite features supported in the specification at, for example, page 8, lines 15-19 and page 13, lines 14-16. No new matter is added by any of these amendments.

Applicants gratefully acknowledge that the rejections under 35 U.S.C §§102(e) and 112, second paragraph are withdrawn. Applicants also gratefully acknowledge withdrawal of the rejection under 35 U.S.C §103 over U.S. Patent 6,656,919 to Baugh *et al.* ("Baugh") in view of "Role of Ger Proteins in Nutrient and Nonnutrient Triggering of Spore Germination in *Bacillus subtilis*", *J. Bacteriology*, May 2000 (vol. 182, no. 9), pp. 2513-2519 by Paidhungat *et al.* ("Paidhungat") and U.S. Patent 6,506,803 to Baker Jr. *et al.* ("Baker").

Reconsideration based on the following remarks is respectfully requested.

I. Amendment Entry After Final Rejection

Entry of this amendment is proper under 37 CFR §1.116 because the amendments: a) place the application in condition for allowance for all the reasons discussed herein; b) do not raise any new issues requiring further search or consideration; c) place the application in better condition for appeal if necessary; and d) address formal requirements of the Final Rejection and preceding Office Action.

The foregoing amendments do not raise any new issues after Final Rejection.

Accordingly, Applicants respectfully request entry of this Amendment.

II. Acknowledgement for References of Record

An Information Disclosure Statement with Form PTO-1449 was filed on March 6, 2002. The Form PTO-1449 returned by the Examiner indicates that only the patent references (listed AA-AR) were considered. The Final Office Action states that the other documents (listed CA-CO) were allegedly not available to the Examiner and thus not considered.

Applicants respectfully request the Examiner to consider the back from the Examiner a copy of the Form PTO-1449 initialed to acknowledge the fact that the Examiner has considered all the cited disclosed information. In order to address the Examiner's preference for copies of the references not yet considered, a copy of each other document is attached. The Examiner is requested to initial and return to the undersigned a copy of the subject Form PTO-1449. For the convenience of the Examiner, a copy of that form is also attached.

III. Obviousness Rejection under 35 U.S.C. §103

The Final Office Action rejects claims 1, 8, 10, 22 and 25 as being allegedly obvious under 35 U.S.C. §103(a) over U.S. Patent 3,617,178 to Clouston in view of Paidhungat and Baker. This rejection is respectfully traversed.

Applicants' claims are directed generally, for example, a method for decontaminating contamination containing biological spores using a spore germination composition. In particular, independent claim 1 recites, *inter alia*, contacting "a spore germination composition comprising from about 10 mM to about 150 mM dipicolinic acid and an amount of calcium ions having a one-to-one ratio with the dipicolinic acid effective to cause rapid germination of the spores." Claim 1 further recites, *inter alia*, concurrently applying "a decontaminating solution comprising amine oxide." Applicants respectfully submit that Clouston, Paidhungat and Baker, alone or in combination, do not describe or suggest at least these claimed features. These arguments also apply to claims 4, 5, 7, 10-16 and 23 based on their dependence from claim 1.

Instead, Clouston discloses a method for sterilizing, disinfecting or preserving substances by applying hydrostatic pressure. In particular, Clouston teaches compressing a sample at a pressure between 100 and 20,000 psi to germinate *Bacillus* spores prior to heating. See, *e.g.*, col. 1, lines 34-42, col. 2, lines 1-14 of Clouston. Example 1 teaches a water medium; Examples 2 and 3 describe a solution of 0.07 M potassium mono and dihydrogen phosphate, and Examples 4-6 teach potassium phosphate. There is no teaching or suggestion in Clouston for any form of chemically triggered spore germination. Rather, by using physical conditions, such as compression, Clouston teaches away from Applicants' claimed features of a composition including either dipicolinic acid (DPA) or calcium ions.

Moreover, Paidhungat and Baker fail to compensate for the deficiencies of Baker. Paidhungat teaches tricistronic operons encoding proteins to germinate *Bacillus* and *Clostridium* bacteria. See, *e.g.*, p. 2513, left column, lines 2-15 of Paidhungat. Further, Paidhungat compares efficacy for quintuple-mutant and wild-type spores between nutrient germination through receptor proteins of the *gerA* family and chemical germination using Ca^{2+} – DPA chelate treatment. See, *e.g.*, Abstract, p. 2515, right column, lines 22-35 and p. 2516, left column, lines 5-11 and right column, lines 36-47 of Paidhungat. However, there is no teaching or suggestion in Paidhungat for decontaminating the spore contamination, thereby negating any motivation by an artisan of ordinary skill to combine the calcium ion and DPA germination of Paidhungat with the pressure application of Baker.

Additionally, Baker teaches a process to inactivate microorganisms using an oil-in-water emulsion. Specifically, Baker discloses emulsions having an aqueous phase, an oil phase and surfactants, with the oil phase including oil and an organic solvent. See, *e.g.*, col. 10, lines 46-49 of Baker. Also, Baker teaches exemplary emulsions that include a lysogenic nanoemulsion of bicomponent triton tri-n-butyl phosphate (BCTP). See *e.g.*, col. 6, lines 20-30, 14, lines 14-19 of Baker. Not only does Baker lack the spore germination composition of Applicants' claims, but Baker also fails to teach or suggest a decontaminating solution that includes amine oxide. As a result, the combined teachings of Clouston, Paidhungat and Baker do not describe or suggest Applicants' claimed features.

Applicants assert that the Examiner's allegation that it would have been obvious to one of ordinary skill in the art to adjust "particular working conditions... is deemed merely a matter of judicious selection and routine optimization of a result-effective parameter" (March 5, 2004 Office Action, p. 6, lines 26-29) is merely a conclusory statement, and that no support for such a statement has been provided. When relying on what is asserted to be general knowledge to negate patentability, that knowledge must be articulated and placed on the record. Applicants respectfully assert that the applied references, whether cited alone or in any combination, fail to teach or suggest the features directed to the spore germination composition and decontaminating solution, any defined quantities notwithstanding. Providing only conclusory statements when dealing with particular combinations of prior art in specific claims cannot support an assertion of obviousness. *In re Lee*, 61 USPQ 2d 1430, 1434-35 (Fed. Cir. 2002).

Thus, there is no proper motivation to combine features related to the high pressure application of Clouston with the tricistronic operons of Paidhungat and the oil-containing emulsion of Baker established in the Final Office Action, which instead relies on previous arguments presented in the March 5, 2004 Office Action at pp. 4-6. However, as explained *supra*, the teachings of the applied references are not sufficiently related, whether in objective or technique, to enable one of ordinary skill to combine their teachings, even based on alternate advantages from those that could be derived from reading Applicants' specification. Even assuming that motivation to combine the applied references is established, the combination fails to teach or suggest Applicants' claimed features.

A *prima facie* case of obviousness for a §103 rejection requires satisfaction of three basic criteria: there must be some suggestion or motivation either in the references or knowledge generally available to modify the references or combine reference teachings, a reasonable expectation of success, and the references must teach or suggest all the claim limitations. See MPEP §706.02(j). Applicants submit that the Final Office Action fails to satisfy these requirements with Clouston, Paidhungat and Baker.

For at least these reasons, Applicants respectfully assert that the independent claim is patentable over the applied references. The dependent claims are likewise patentable over the applied references for at least the reasons discussed, as well as for the additional features they recite. Consequently, all the claims are in condition for allowance. Thus, Applicants respectfully request that the rejection under 35 U.S.C. §103 be withdrawn.

IV. Conclusion

In view of the foregoing amendments and remarks, Applicants respectfully submit that this application is in condition for allowance. Favorable reconsideration and prompt allowance are earnestly solicited.

Applicants : Amanda S. Schilling *et al.*
Serial No. : 10/090,798
Filed : March 6, 2002
Page : 8 of 8

Attorney Docket No.: Navy Case 83202

Should the Examiner believe that anything further is desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact Applicants' undersigned representative at the telephone number listed below.

Respectfully submitted,



Gerhard W. Thielman
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Attachments:

Copy of earlier filed PTO-1449
Copy of "other documents" (references CA-CO)

Date: November 8, 2005

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FORM PTO 1449 (REV. 2-32)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTY DOCKET NO.: NC# 83,202		SERIAL NO.:	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				APPLICANT: Schilling, A. et al.			
(Use several sheets if necessary)				FILING DATE		GROUP	
U.S. PATENT DOCUMENTS							
EXAMINER INITIAL		DOCUMENT N UMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	3,957,695	5/18/1976	Davies et al.	510	348	
	AB	4,076,653	2/28/1978	Davies et al.	510	348	
	AC	5,236,612	8/17/1993	Rahman et al.	510	505	
	AD	5,352,387	10/4/1994	Rahman et al.	510	496	
	AE	5,358,656	10/25/1994	Humphreys et al.	510	433	
	AF	5,385,685	1/31/1995	Humphreys et al.	510	119	
	AG	5,360,573	11/1/1994	Smith et al.	252	186.39	
	AH	5,389,279	2/14/1995	Au et al.	424	70.19	
	AI	5,484,555	1/16/1996	Schepers	8	137	
	AJ	5,412,118	5/2/1995	Vermeer et al.	510	127	
	AK	5,616,280	4/1/1997	Moore et al.	252	186.29	
	AL	5,795,730	8/18/1998	Tautvydas	435	31	
	AM	5,863,882	1/26/1999	Lin et al.	510	397	
	AN	5,908,707	6/1/1999	Cabell et al.	428	537.5	
	AO	6,077,317	6/20/2000	Murphy	8	137	
	AP	6,121,165	9/19/2000	Mackey et al.	442	84	
	AQ	6,165,965	12/26/2000	Schalitz et al.	510	384	
	AR	6,270,878	8/7/2001	Wegele et al.	428	195	
FOREIGN PATENT DOCUMENTS							
	BA						
OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, etc.)							
	CA	Atrih, A., P. Zollner, G. Allmaier, M. P. Williamson and S. J. Foster. 1998. Peptidoglycan structural dynamics during germination of <i>Bacillus subtilis</i> 168 endospores. J. Bacteriol. 180: 4603-12.					
	CB	Behravan, J., H. Chirakkal, A. Masson and A. Moir. 2000. Mutations in the gerP locus of <i>Bacillus subtilis</i> and <i>Bacillus cereus</i> affect access of germinants to their targets in spores. J. Bacteriol. 182:1987-94.					

	CC	Black, S. H. and P. Gerhardt. 1961. Permeability of Bacterial Spores III. Permeation Relative to Germination. J. Bacteriol. 88:301-308.
	CD	Doi, R. H. 1989. Sporulation and germination. In <i>Bacillus</i> . Colin R. Harwood, ed. Plenum Press: NY. p. 169-215.
	CE	Foster, S. J. and K. Johnstone. 1990. Pulling the trigger: the mechanism of bacterial spore germination. Molecular Microbiology (4):137-41.
	CF	Johnstone, K. 1994. The trigger mechanism of spore germination: current concepts. Journal of Applied Bacteriology Symposium Supplement. 76:17S-24S.
	CG	Koshikawa, T., T. C. Beaman, H. S. Pankratz, S. Nakashio, T. R. Corner and P. Gerhardt. 1984. Resistance, germination, and permeability correlates of <i>Bacillus megaterium</i> spores successively divested of integument layers. J. Bacteriol. 159:624-32.
	CH	Moir, A. and D.A. Smith. 1990. The genetics of bacterial germination. Annu. Rev. Microbiol. 44:531-53.
	CI	Moir, A., E.H. Kemp, C. Robinson, and B.M. Corfe. 1994. The genetic analysis of spore germination. Journal of Applied Bacteriology Symposium Supplement. 76: 9S-16S.
	CJ	Nicholson, W.L. and P. Setlow. 1990. Sporulation, germination and outgrowth. In Molecular Biological Methods for <i>Bacillus</i> . C. R. Harwood and S. M. Cutting, eds. John Wiley and Sons: NY. p. 391-429.
	CK	Paidhungat, M, B. Setlow, A. Driks, and P. Setlow. 2000. Characterization of spores of <i>Bacillus subtilis</i> which lack dipicolinic acid. J. Bacteriol. 182(19):5505-5512.
	CL	Sacks, L.E. 1990. Chemical germination of native and cation-exchanged bacterial spores with trifluoperazine. Appl. Environ. Microbiol. 56:1185-7.
	CM	Sanchez-Salas, J.L., and P. Setlow. 1993. Proteolytic processing of the protease which initiates degradation of small, acid-soluble proteins during germination of <i>Bacillus subtilis</i> spores. J. Bacteriol. 175:2568-77.
	CN	Wax, R. and Ernst Freese. 1968. Initiation of the germination of <i>Bacillus subtilis</i> spores by a combination of compounds in place of L-alanine. J. Bacteriol. 95(2):433-438.
	CO	Wuytack, E.Y., S. Boven and C. W. Michiels. 1998. Comparative Study of Pressure-Induced Germination of <i>Bacillus subtilis</i> Spores at Low and High Pressures. Appl. Environ. Microbiol. 64: 3220-3224.

EXAMINER

DATE CONSIDERED

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. include copy of this form with next communication to applicant .

Peptidoglycan Structural Dynamics during Germination of *Bacillus subtilis* 168 Endospores

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Peptidoglycan structural dynamics during endospore germination of *Bacillus subtilis* 168 have been examined by mucopeptide analysis. The first germination-associated peptidoglycan structural changes are detected within 3 min after the addition of the specific germinant L-alanine. We detected in the spore-associated material new mucopeptides which, although they have slightly longer retention times by reversed-phase (RP)-high-pressure liquid chromatography (HPLC) than related ones in dormant spores, show the same amino acid composition and molecular mass. Two-dimensional nuclear magnetic resonance (NMR) analysis shows that the chemical changes to the mucopeptides on germination are minor and are probably limited to stereochemical inversion. These new mucopeptides account for almost 26% of the total mucopeptides in spore-associated material after 2 h of germination. The exudate of germinated spores of *B. subtilis* 168 contains novel mucopeptides in addition to those present in spore-associated material. Exudate-specific mucopeptides have longer retention times, have no reducing termini, and exhibit a molecular mass 20 Da lower than those of related reduced mucopeptides. These new products are anhydro-mucopeptides which are generated by a lytic transglycosylase, the first to be identified in a gram-positive bacterium. There is also evidence for the activity of a glucosaminidase during the germination process. Quantification of mucopeptides in spore-associated material indicates that there is a heterogeneous distribution of mucopeptides in spore peptidoglycan. The spore-specific residue, muramic δ -lactam, is proposed to be a major substrate specificity determinant of germination-specific lytic enzymes, allowing cortex hydrolysis without any effect on the primordial cell wall.

The extreme heat resistance of dormant bacterial endospores has made them an important problem in the production of safe foodstuffs (3). The spore cell wall peptidoglycan is considered to play a major role in the maintenance of heat resistance and dormancy (6). *Bacillus subtilis* spore peptidoglycan is composed of two layers. A thin, inner layer called the primordial cell wall retains the basic vegetative cell peptidoglycan structure. The primordial cell wall represents 2 to 4% of the total endospore peptidoglycan, is not digested during germination, and serves as the initial cell wall during outgrowth (2, 5, 25, 29). The outer thick layer of peptidoglycan, known as the cortex, is characterized by several unique spore-specific features. Approximately 50% of the muramic acid residues in the glycan strands are present in the δ -lactam form (2, 24). Muramic acid side chains are composed of 26 and 23% of tetrapeptide and single L-alanine, respectively (2).

Despite their extreme dormancy and thermostability, bacterial endospores retain an alert sensory mechanism enabling them to respond within minutes to the presence of specific germinants. Spores of *B. subtilis* respond to at least two different types of germinative stimuli: (i) L-alanine and (ii) a combination of L-asparagine, glucose, fructose, and KCl (AGFK) (34). The germination response is initiated by the interaction of a receptor protein with specific germinants which triggers the loss of spore-specific properties and the transformation of a dormant resistant bacterial spore into a metabolically active

vegetative cell. The germination process is characterized by sequential, interrelated biochemical events. The specific hydrolysis of peptidoglycan in the spore cortex layer is an essential event in germination (2, 25). Its degradation removes the physical constraints of the cortex and allows core expansion and outgrowth (9, 25). As a consequence of cortex hydrolysis, peptidoglycan fragments can be detected in the germination exudate (13, 33).

A number of bacterial spore germination-specific cortex-lytic enzymes (GSLEs) have been reported to be involved in cortex hydrolysis (9, 18-20). A gene homologous to that encoding the GSLE from *Bacillus cereus* has been identified and inactivated in *B. subtilis*, and the resulting mutant germinates more slowly than the wild type (22). Recently a germination-specific muramidase isolated from a germination extract of *Clostridium perfringens* S40 has been purified and characterized (4).

GSLEs have a high substrate specificity, requiring intact spore cortex for activity (9, 23). The muramidase from *C. perfringens* S40, however, hydrolyzes cortical fragments but has a strict requirement for the presence of the muramic δ -lactam residues (4). Thus, the GSLEs are highly specialized and may exist as proforms which are specifically activated during germination (9).

Very little is known about the mechanism by which the cortex is hydrolyzed during germination and the autolytic enzymes involved. Mucopeptide analysis provides a method for fine chemical structural determination of spore cortex (2, 24, 25). In this paper, we report the use of mucopeptide analysis to determine the peptidoglycan structural dynamics which occur during spore germination of *B. subtilis* 168 and the evidence for a number of different enzyme activities.

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MATERIALS AND METHODS

Bacterial strains and sporulation conditions. All *B. subtilis* 16S strains used in this study are in the HR background (2). Specific mutations were transferred into HR by transformation with donor chromosomal DNA (1). Spores were prepared and stored as previously described (2).

Spore germination. Purified spores were heat activated in distilled water at 70°C for 45 min. Activated spores were quickly cooled in ice and used within 1 h for germination experiments. Spores were suspended at a final concentration of 9 to 11 mg/ml in 30 mM potassium phosphate buffer (pH 7) and prewarmed for 15 min to 37°C before addition of L-alanine to a final concentration of 1 mM. Continuous monitoring of germination was carried out by recording the decrease of A_{600} (9).

Determination of the loss of heat resistance during germination. Germinating spore samples (100 μ l) were added immediately to 900 μ l of 10 mM D-alanine and incubated for 25 min at 70°C. After cooling, viability was measured by serial dilution and plate counting on nutrient agar (8).

Preparation of spore-associated peptidoglycan. Germinating spore samples (3 ml) were added directly to 6 ml of propan-1-ol (prewarmed to 80°C) and incubated for 15 min at 80°C to stop germination. Spores were recovered by centrifugation (14,000 \times g, 8 min, room temperature), and resuspended in 1 ml of 50 mM Tris-HCl (pH 7)–4% (wt/vol) sodium dodecyl sulfate–30 mM dithiothreitol–2 mM EDTA, boiled for 16 min, and then incubated at 37°C for 40 min. Peptidoglycan-containing insoluble material was recovered by centrifugation (14,000 \times g, 8 min, room temperature) and washed by repeated resuspension and centrifugation with warm (37°C) distilled water until free of sodium dodecyl sulfate. Samples were finally resuspended in MilliQ water (18 M Ω cm) and stored at –20°C.

Preparation of germination exudate. For the analysis of the germination exudate, 3-ml aliquots of germinating spore samples were centrifuged (14,000 \times g, 8 min, room temperature), and the supernatant was treated for 3 min at 100°C to inactivate the cortex lytic enzyme(s). The supernatant was freeze-dried, resuspended in 1 ml of MilliQ water, and stored at –20°C.

RP-HPLC, amino acid analysis, and MS. Spore-associated peptidoglycan was digested with Cellosyl and reduced with sodium borohydride as previously described (2). Germination exudate was reduced with sodium borohydride (3.3 mg/ml) after Cellosyl digestion. Reverse-phase high-pressure liquid chromatography (RP-HPLC), desalting, amino acid analysis, and mass spectrometry (MS) were performed as previously reported (2).

Gel filtration of germination exudate samples. Freeze-dried germination exudate samples were resuspended in MilliQ water and applied to a TSK SW2000 gel filtration column (7.8 mm by 30 cm). The column was eluted with 10 mM sodium phosphate (pH 6.5) at 0.3 ml/min. The eluate was then desalted and analyzed as described above.

Nuclear magnetic resonance (NMR) analysis of mucopeptides. Samples of ca. 1 mM mucopeptide were prepared in 90% H₂O–10% D₂O, and studied at 19 to 35°C on a Bruker DRX-500 spectrometer. Spectra were assigned by using two-dimensional (2D) correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), and rotating frame nuclear Overhauser effect spectroscopy (ROESY), which were acquired by using spectral widths of 12,500 Hz in t_2 and 5,000 Hz in t_1 over 256 complex points with quadrature detection using the States-TPPI scheme. Mixing times for both TOCSY and ROESY were 100 ms. Spectra were processed by using Felix 97.0 (Molecular Simulations, Inc., San Diego, Calif.).

RESULTS

Changes in spore-associated peptidoglycan structure during germination. To avoid possible loss of mucopeptides from germinated spores during spore extraction, only the first detergent treatment of the previously derived protocol was used (2). After this extraction, almost 97% of the peptidoglycan was solubilized after Cellosyl digestion. The RP-HPLC profiles of mucopeptides from dormant and germinated spore-associated material (2 h after addition of L-alanine) are shown in Fig. 1A and B. During germination, >60% of the original A_{600} was lost by the spore population over 2 h. The major germination-associated changes in mucopeptide profile comprised a decrease in the muramic δ -lactam-containing mucopeptides, which are characteristic of the spore cortex (e.g., mucopeptides 6, 7, 10, and 11), and the appearance of seven novel mucopeptides (Fig. 1B, mucopeptides G1 to G7).

RP-HPLC analysis of the germination exudate. The RP-HPLC profile of the germination exudate, after Cellosyl digestion, revealed the appearance of several potential mucopeptides (Fig. 1C). Nearly all the spore-associated mucopeptides were also found in the exudate (e.g., mucopeptides 6, 7, 10, and

11 [Fig. 1B and C]). However, G9, G10, G11, G12, and G13 are germination exudate-specific products. Approximately the same amounts of products labeled X were found in the germination exudate whether digested with Cellosyl or not (Fig. 1C and D). The resolved X peaks are not peptidoglycan derived since they do not contain amino acids or amino sugars (results not shown). The novel exudate-specific products G9, G10, and G13 were also resolved without Cellosyl digestion (Fig. 1D), but their amounts increased following digestion (Fig. 1C). Omission of borohydride reduction did not affect the peak shapes or retention times of products G9, G10, G12, and G13 (Fig. 1C and D and results not shown).

Molecular weight determination of native peptidoglycan fragments in the germination exudate. The profiles of the germination exudate with (Fig. 1C) or without (Fig. 1D) Cellosyl digestion revealed that most of the peptidoglycan is released in the form of fragments too large to be resolved by RP-HPLC. Gel filtration was used to purify the native fragments (results not shown). Peptidoglycan-derived material was shown to consist of several molecular species, ranging from m/z 1,758 to 5,537.5.

Germination by AGFK and the role of peptidoglycan and protein biosynthesis. Germination in the presence of AGFK led to mucopeptide flux comparable to that in L-alanine (results not shown). Also, the addition of chloramphenicol (100 μ g/ml) or penicillin G (100 μ g/ml) to the germination mix had no significant effect on the mucopeptide profiles (results not shown). Therefore, cortex modification and hydrolysis are common to different germinants and are not due to the synthesis of new enzymes or peptidoglycan during germination.

Characterization of the novel spore-associated mucopeptides. All of the germination-specific mucopeptides (Fig. 1B) were purified, characterized by amino acid analysis and MS (Table 1), identified, and quantified (Table 2). All are peptidoglycan derived and have the same basic composition as dormant spore mucopeptides (Table 2). Mucopeptides G1 to G7 all have their equivalents in the dormant spore, to which they are ostensibly identical in terms of amino acids and MS (Tables 1 and 2, mucopeptides 6, 7, 10, 11, 13, 20, and 21, respectively) (2). The germination-specific mucopeptides all, however, show a characteristic increase in retention times over their dormant spore counterparts (Fig. 1A and B). The germination-specific mucopeptides all have reducing termini and are unaffected by HF (48% [vol/vol], 24 h, 0°C), HCl (4 M, 15 min, 35°C), or desalting treatment prior to separation by RP-HPLC compared to the equivalent dormant spore mucopeptides (results not shown). One-dimensional NMR clearly showed the absence of amidation in the novel mucopeptides (results not shown); amidation would cause a mass change of only one mass unit and thus be hard to detect by MS. Further analysis by 2D NMR showed that corresponding pairs of normal and germination-specific mucopeptides have very similar chemical shifts and ROESY spectra (Fig. 2 and Table 3), indicating that the covalent structures of the novel mucopeptides are very similar to those of their parent mucopeptides. In particular, nuclear Overhauser enhancements between sugars confirmed that there is no alteration in linkage on germination. Thus, the germination-associated change is a subtle modification that does not affect the gross structure and is most likely a change in the stereochemistry at one or more chiral centers. After 2 h of germination, the novel mucopeptides (G1 to G7) constitute 25.8% of the total spore-associated material.

The novel germination-associated mucopeptides are not the result of alanine racemase activity, as they still appeared during germination of *B. subtilis* 1A288 (*amyE dal-1 metB5 sacA321*), which strictly requires D-alanine for growth. Also, the addition

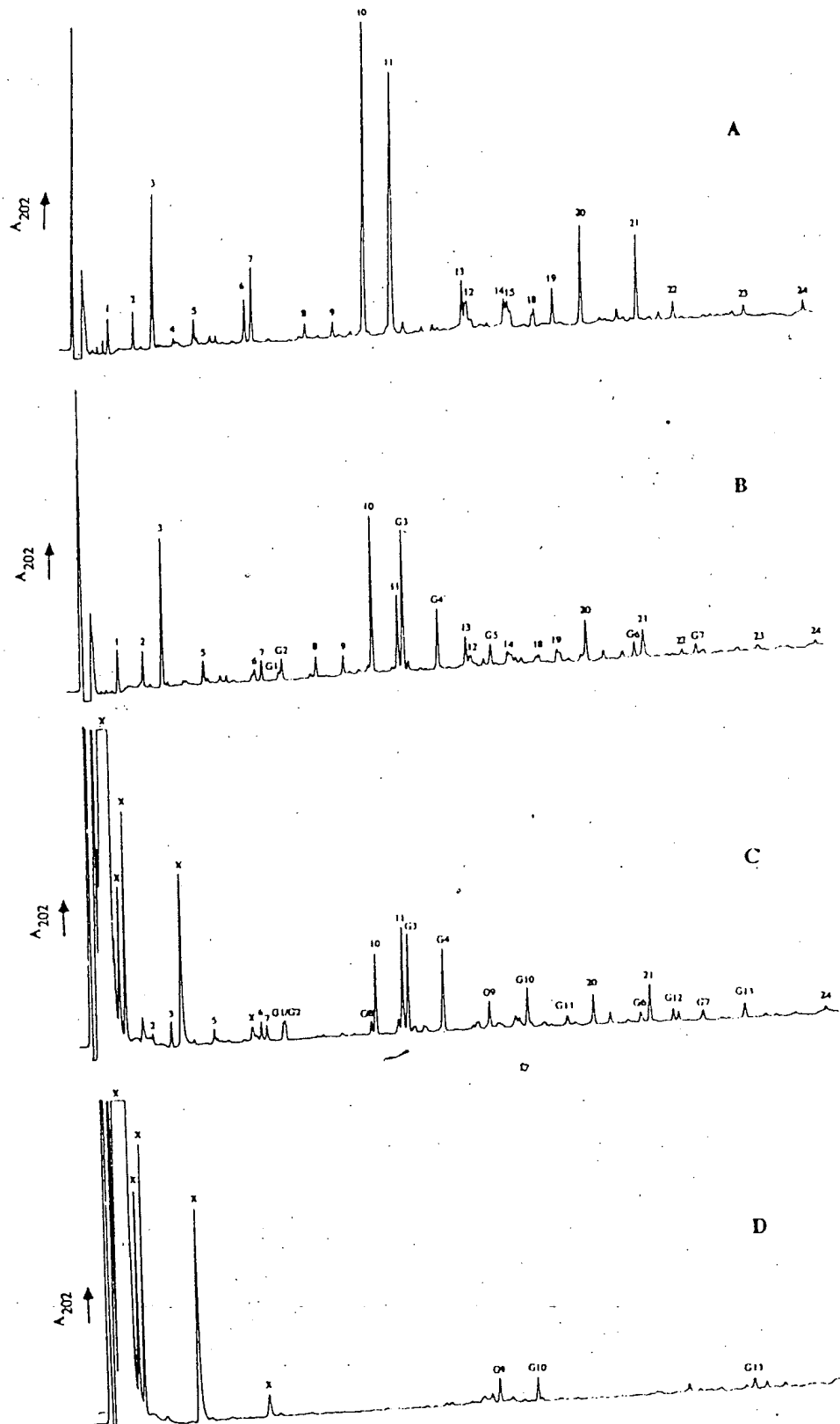


FIG. 1. Analysis of mucopeptides by RP-HPLC during germination (120 min) of *B. subtilis* 168 HR spores. Mucopeptide-containing samples were separated by RP-HPLC, and the A_{202} of the eluates was monitored. (A) Dormant spore-associated material; (B) germinated spore-associated material; (C) germination exudate; (D) germination exudate (no Cellosyl digestion or reduction).

of *O*-carbamyl-D-serine (a potent inhibitor of alanine racemase) (26) at 100 $\mu\text{g}/\text{ml}$ had no effect on germination kinetics or mucopeptide modification.

Characterization of the novel germination exudate-specific products. All products labeled in Fig. 1C are peptidoglycan

derived except those lettered X, which are also found in the exudate without Cellosyl digestion (Fig. 1D). Germination exudate-specific mucopeptides G9 to G13 (Fig. 1C) have the same amino acid analysis but a characteristic mass deviation of -20 Da determined by matrix-assisted laser desorption-ioniza-

TABLE 1. Calculated and observed m/z values for sodiated and deprotonated molecular ions of new mucopeptides identified during *B. subtilis* 168 HR germination

Mucopeptide ^a	Ion	m/z		Δm (Da) ^b	Error (%)	Mucopeptide composition ^d					
		Observed	Calculated			Glc	Mur	δ -Mur	Glu	Ala	Dmp
G1	[M + Na] ⁺	996.4	1,011.0	-14.6		2	1	1	0	1	0
	[M - H] ⁻	972.5	987.0	-14.5							
G2	[M + Na] ⁺	1,369.6	1,383.3	-13.7		2	1	1	1	2	1
	[M - H] ⁻	1,345.7	1,359.3	-13.6							
G3	[M + Na] ⁺	1,384.4	1,383.3	1.1	0.07	2	1	1	1	2	1
	[M - H] ⁻	1,359.6	1,359.3	0.3	0.02						
G4	[M + Na] ⁺	1,009.6	1,011.0	-1.4	-0.13	2	1	1	0	1	0
	[M - H] ⁻	986.3	987.0	-0.7	-0.07						
G5	[M + Na] ⁺	2,307.5	2,307.2	-0.3	-0.01	3	2	1	2	4	2
	[M - H] ⁻	2,283.1	2,283.2	-0.1	-0.004						
G6	[M + Na] ⁺	1,802.5	1,801.7	-0.8	-0.04	3	1	2	1	2	1
	[M - H] ⁻	1,778.3	1,777.7	-0.6	-0.03						
G7	[M + Na] ⁺	1,429.9	1,429.4	0.5	0.03	3	1	2	0	1	0
	[M - H] ⁻	1,405.5	1,405.4	0.1	0.007						
G8	[M + Na] ⁺	1,178.4	1,383.3	-204.9		1	1	1	1	2	1
	[M - H] ⁻	1,155.3	1,359.3	-204.0							
G9	[M + Na] ⁺	1,363.4	1,383.3	-19.9		2	1	1	1	2	1
	[M - H] ⁻	1,339.1	1,359.3	-20.2							
G10	[M + Na] ⁺	990.5	1,011.0	-20.5		2	1	1	0	1	0
	[M - H] ⁻	966.6	987.0	-20.4							
G11	[M + Na] ⁺	990.4	1,011.0	-20.6		2	1	1	0	1	0
	[M - H] ⁻	966.1	987.0	-20.9							
G12	[M + Na] ⁺	1,781.3	1,801.7	-20.4		3	1	2	1	2	1
	[M - H] ⁻	1,757.1	1,777.7	-20.6							
G13	[M + Na] ⁺	1,409.3	1,429.4	-20.1		3	1	2	0	1	0
	[M - H] ⁻	1,385.1	1,405.4	-20.3							

^a Numbered as indicated in Fig. 1.^b Difference between observed and calculated sodiated or deprotonated molecular mass values. Boldface numbers denote deviations where the calculated values are the most likely combinations of the substituent components.^c Calculated as [(observed mass - calculated mass)/calculated mass] \times 100.^d Glc, *N*-acetylglucosamine; Mur, *N*-acetylmuramic acid; δ -Mur, muramic δ -lactam.

tion (MALDI) reflector time-of-flight MS compared to dormant spore mucopeptides 10, 11, 20, and 21, respectively (Tables 1 and 2) (2). As G11 has a longer retention time than G10, it may be derived from the germination-specific spore-associated mucopeptide G4 (Fig. 1C). The germination exudate-specific mucopeptides (G9 to G13) all have longer retention times than their related spore mucopeptides (Fig. 1C). Omission of sodium borohydride reduction prior to RP-HPLC led to loss of resolution and alterations in retention time of all mucopeptides apart from G9 to G13 (results not shown). All of the features of G9 to G13 suggest that they have a 1-6 anhydromuramic acid moiety. The positive- and negative-ion MALDI mass spectrum of mucopeptide G12, which is the largest mass spectrometrically determined anhydro-mucopeptide in *B. subtilis*, is shown in Fig. 3. The peak at m/z 1,781.3 corresponds to the [M + Na]⁺ molecular ion (Fig. 3A). Several satellite peaks were detected and corresponded to [M + H]⁺, [M + 2Na - H]⁺ and [M + 3Na - H]⁺ molecular ions. Further, in the positive-ion mode an intense fragment ion at m/z 1,558.2 ([M + H - GlcNAc]⁺) was determined. In the negative-ion mode, the base peak at m/z 1,757.1 corresponded to the molecular ion [M - H]⁻ (Fig. 3B). The lack of 20 Da corresponds to the loss of one molecule of water between carbon 1 and carbon 6 of the *N*-acetylmuramic acid and the two hydrogens which would have been gained by sodium borohydride reduction. Anhydro-mucopeptides have been found in gram-negative bacteria and are known for their hydrophobic character and acid lability (11, 12). These mucopeptides are produced by the action of a lytic transglycosylase (12). G9 to G13 account for almost 19% of the total mucopeptides in the germination

exudate (Table 2). Interestingly, almost 55% of the dominant anhydro-mucopeptides G9, G10, and G13 are also present in the exudate without Cellosyl digestion (Fig. 1D).

Mucopeptide G8 is a trisaccharide tetrapeptide (Fig. 1C; Tables 1 and 2); the missing 204 Da corresponds to an *N*-acetylglucosamine moiety. G8 is likely to have been generated by the activity of an *N*-acetylglucosaminidase during germination. G8 accounts for only 1.4% of total exudate mucopeptides, and the glucosaminidase activity is therefore minor compared to the lytic transglycosylase activity.

Muramidase activity during germination? To determine whether a germination-specific muramidase is involved in cortex hydrolysis, as reported for *C. perfringens* (4), the germination exudate RP-HPLC profiles were examined after various treatments. Only anhydro-mucopeptides were detected by RP-HPLC when non-Cellosyl-digested exudate was separated with or without sodium borohydride reduction (Fig. 1D and results not shown). When the germination exudate was reduced, digested with Cellosyl, and analyzed by RP-HPLC, an increase in anhydro-mucopeptides and the appearance of nonreduced tetrasaccharide alanine and tetrasaccharide tetrapeptide were noted (the nonreduced mucopeptides have retention times different from those of the reduced forms). However, when this sample was reduced again after Cellosyl digestion, the RP-HPLC profile was comparable to that in Fig. 1C. This clearly indicates that there is not a significant amount of muramic acid residues with free reducing termini in the native germination exudate (which would result from muramidase activity). Thus, it is unlikely that gross muramidase activity is involved in *B. subtilis* cortex hydrolysis during germination.

TABLE 2. Muropeptide identities and quantification^a

Muropeptide	Identity	Mol%		
		DM	SAM	GE
1	Disaccharide tripeptide	3.3	6.1	
2	Disaccharide alanine	5	7	2.3
3	Disaccharide tetrapeptide	13.8	18.9	5.7
4	Tetrasaccharide alanine with open lactam	1.0		
5	Tetrasaccharide tetrapeptide with open lactam	2.6	5.0	3.0
6	Tetrasaccharide alanine with a reduced lactam	4.6	1.3	4
7	Tetrasaccharide tetrapeptide with a reduced lactam	4.8	2	1.7
8	Disaccharide tripeptide disaccharide tetrapeptide	0.8	1.3	
9	Disaccharide tetrapeptide disaccharide tetrapeptide	0.7	1.0	
10	Tetrasaccharide tetrapeptide	20.1	9.8	8.8
11	Tetrasaccharide alanine	22.0	8.5	13.8
12	Hexasaccharide tetrapeptide with one reduced lactam	1.5	0.6	
13	Disaccharide tetrapeptide tetrasaccharide tetrapeptide	2	1.7	
14	Hexasaccharide alanine with one reduced lactam	0.8	0.6	
15	Hexasaccharide alanine with one reduced lactam	1.1		
18	Hexasaccharide alanine with three acetylations and one reduced lactam	1.6	1.9	
19	Tetrasaccharide tetrapeptide tetrasaccharide tetrapeptide	0.9	0.6	
20	Hexasaccharide tetrapeptide	6	3.3	4.6
21	Hexasaccharide alanine	5.6	3.5	5.6
22	Tetrasaccharide tetrapeptide hexasaccharide tetrapeptide	0.5	0.3	
23	Octasaccharide tetrapeptide	0.6	0.4	
24	Octasaccharide alanine	0.7	0.5	0.4
G1	Tetrasaccharide alanine with a reduced lactam		1.1	1.4
G2	Tetrasaccharide tetrapeptide with a reduced lactam		3	2.2
G3	Tetrasaccharide tetrapeptide		9.8	10.7
G4	Tetrasaccharide alanine		8.0	12.5
G5	Disaccharide tetrapeptide tetrasaccharide tetrapeptide		0.9	
G6	Hexasaccharide tetrapeptide		1.5	1.3
G7	Hexasaccharide alanine		1.4	1.8
G8	Trisaccharide tetrapeptide			1.4
G9	Anhydro-tetrasaccharide tetrapeptide			5
G10	Anhydro-tetrasaccharide alanine			8.5
G11	Anhydro-tetrasaccharide alanine			1.5
G12	Anhydro-hexasaccharide tetrapeptide			1.4
G13	Anhydro-hexasaccharide alanine			2.4

^a Peptidoglycan was from dormant spores (DM) of *B. subtilis* 168 HR, spore-associated material after 120 min of germination (SAM), and germination exudate after 120 min of germination (GE).

Kinetics of peptidoglycan structural dynamics, and other biochemical events, during germination. The kinetics of biochemical events occurring during germination were examined to determine their sequential interrelationships. The dominant germination-associated muropeptides, G3 and G4 (Fig. 1B; Table 2), were detected 3 min after addition of L-alanine and increased throughout germination (Fig. 4). However, loss of heat resistance and absorbance were measurable within 1 min.

Spore-associated muropeptides were quantified throughout germination. The percentage decreases of total muropeptides containing hexasaccharides and tetrasaccharides were 42 and 39%, respectively, within 30 min (Fig. 5). Disaccharide alanine- and disaccharide tetrapeptide-containing muropeptides decreased at a lower rate; only 18% of the initial amount was lost over the same period (Fig. 5). The loss of disaccharide tripeptide-containing material (muropeptides 1 and 8) during germination was minimal (Fig. 5). The trends in muropeptide dynamics continued over 2 h of germination (data not shown). Muropeptide quantification of the germination exudate (Table 2) confirms the differential muropeptide loss from the germinating spores. Indeed, tetrasaccharide- and hexasaccharide-containing muropeptides constitute the major products found in the exudate (Table 2). The relative percentage increase of disaccharide-containing material in the spore-associated peptidoglycan during germination (Table 2) is due not to biosyn-

thesis of these muropeptides but rather to the greater relative decrease in the muropeptides containing muramic δ -lactam residues (Fig. 5).

Germination of *cwlD* and other germination mutants. Germination of AA107 (*cwlD*) resulted in a 40% decrease in A_{600} over 2 h, but no structural alterations of the spore peptidoglycan occurred over this time period. The cortex of this strain has no muramic δ -lactam residues (2, 25). Spores of strains AA114 (*gerD* [32]) and AA115 (*gerB* [21]) had dormant spore peptidoglycan structures comparable to that of HR (wild type) except that muropeptides with single L-alanine substituents were present at lower levels in AA115 (*gerB*). AA115 (*gerB*) germinated in L-alanine showed the same peptidoglycan dynamics as HR (wild type) and no changes in the presence of AGFK (as expected, as the mutant cannot respond specifically to the AGFK germinants). AA114 (*gerD* [32]) germinated slowly with 10 mM L-alanine and 10 mM KCl (35% loss of A_{600} after 4 h) and showed the same structural changes as HR (wild type) but at a lower rate.

DISCUSSION

Specific cortex hydrolysis by the action of a GSLE is an essential step during endospore germination, as its removal allows spore core expansion and outgrowth (10, 13, 30). This

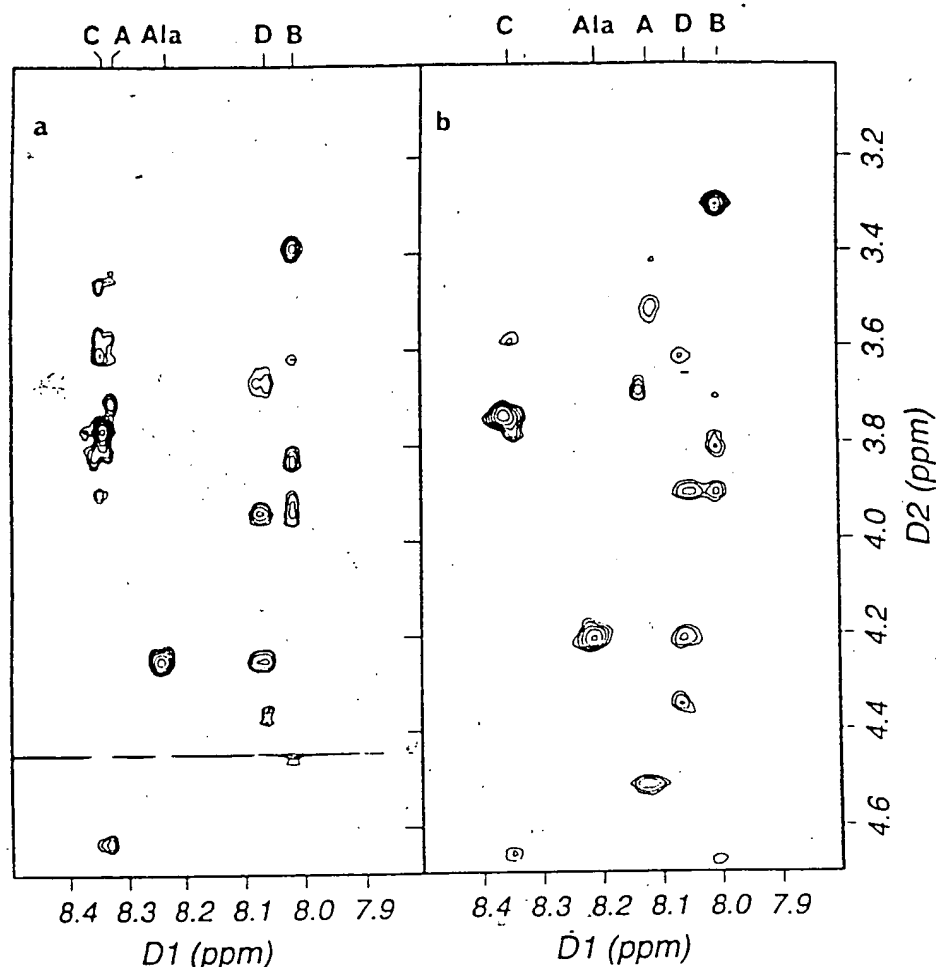


FIG. 2. Portions of the ROESY spectra of the corresponding dormant and germination-associated tetrasaccharide alanine muropeptides 11 and G4 (a and b, respectively). The spectra show nuclear Overhauser enhancements between the 2'-amide protons (and alanine amide proton) and other protons in the muropeptides. The protons are labeled at the top with the identity of the saccharide unit (from A at the nonreducing end to D at the reducing end). Chemical shift assignments for these muropeptides are given in Table 3.

finding is corroborated by the fact that the *cwlD* mutant has an altered spore cortex structure and is unable to outgrow and form a colony on a plate (2, 25, 28). This observation led to the suggestion that the muramic δ -lactam residues (missing in

cwlD) are part of the substrate recognition profile of the GSLE (2, 9, 25). However, the mechanism of cortex hydrolysis during germination and the number of enzymes involved have remained obscure.

TABLE 3. NMR chemical shift assignments for the tetrasaccharide alanine muropeptides 11 and G4 (dormant and germinating spore-associated, respectively) (1 mM, 30°C)

Proton	NMR chemical shift assignment ^a (ppm)									
	A		B		C		D		Ala	
	11	G4	11	G4	11	G4	11	G4	11	G4
1'	4.63	4.52	4.73	4.84	4.69	4.66	3.68, 3.73	3.73, 3.67		
2'	3.70	3.71	3.38	3.31	3.76	3.75	4.36	4.41		
3'	3.56	3.42	3.63	3.65	3.43	3.58	3.94	3.96		
Others	3.45	3.53	3.83	3.86	3.59	3.77	3.88	3.84		
	3.80	3.88 ^b	3.91	4.13	3.93	3.91	3.65	3.67		
	3.91	3.88 ^b	3.70	3.70	3.61	3.64	3.80	3.84		
NH	8.33	8.12	8.00	8.01	8.33	8.35	8.05	8.05	8.23	8.21
Methyl	2.07	2.04	1.45	1.44	2.07	2.06	2.06	1.98	1.40	1.37
CH			4.45	4.39					4.22	4.19
Mur Me							1.40	1.37		
Mur CH							4.22	4.19		

^a The saccharide units are labeled from A at the nonreducing end to D at the reducing end; A and C are *N*-acetylglucosamines, B is a muramic δ -lactam, and D is a reduced muramic acid carrying alanine at the 3' position.

^b It was not possible to tell from the NMR spectrum at 3.88 ppm of G4 if this position corresponds to one or two protons.

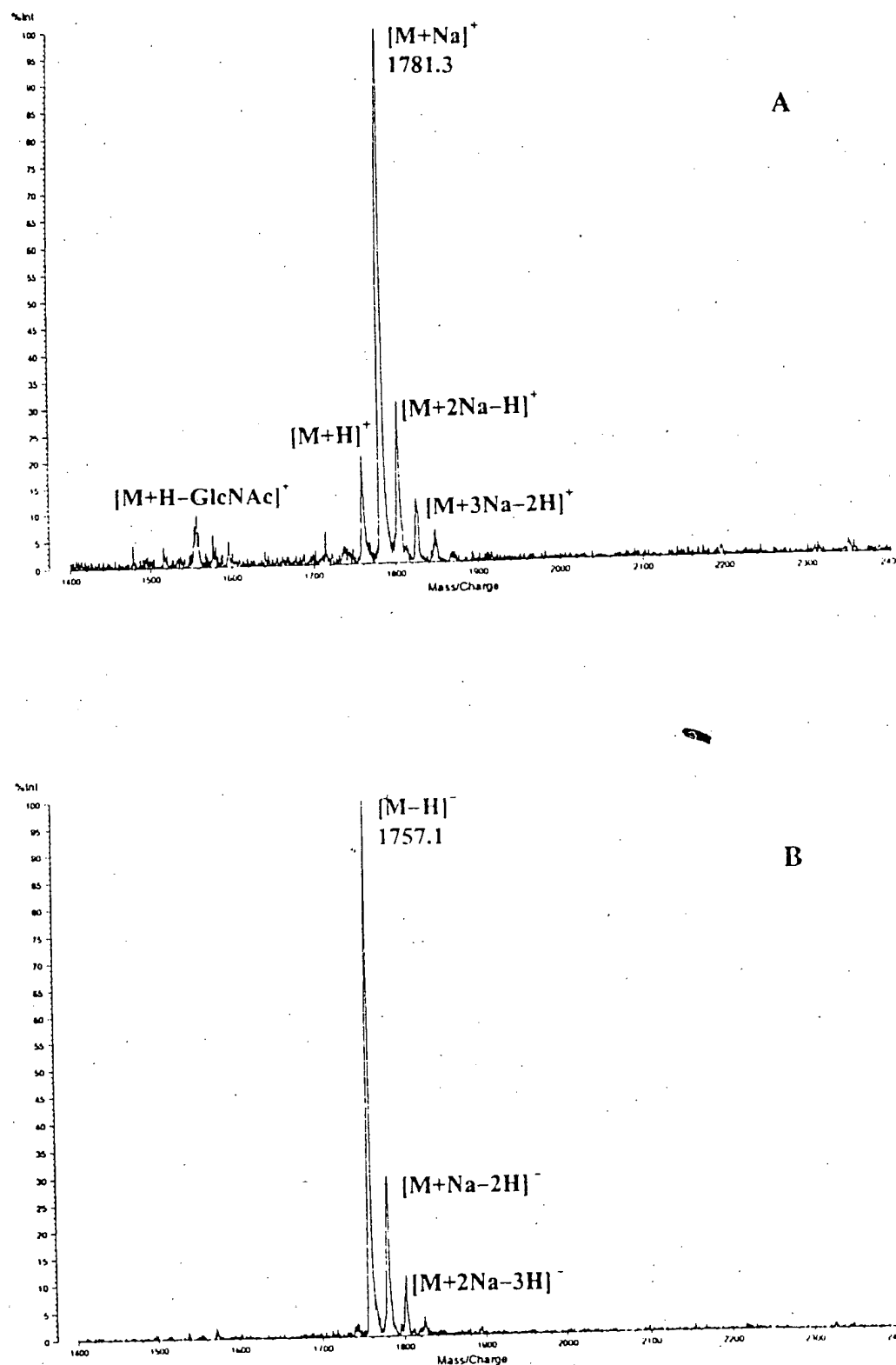


FIG. 3. Positive (A)- and negative (B)-ion MALDI mass spectrum of mucopeptide G12 (Tables 1 and 2; anhydro-hexasaccharide tetrapeptide) obtained in the reflector mode.

Cortex modification as reflected by changes in peptidoglycan structure is initiated within 3 min of addition of the germinant L-alanine. The modification is stable and does not arise from amidation or hydrolytic cleavage, although it is possible that the modified mucopeptides are then marked for hydrolysis by ensuing autolytic enzymes. Alternatively, the modification may

not be essential for germination but rather has a more subtle role. It is clear that the cortex modification is not essential for loss of absorbance or heat resistance, because these changes precede the modification (Fig. 4). Furthermore, spores of the *cw/D* mutant lose heat resistance and partial absorbance on germination, even though cortex modification does not occur

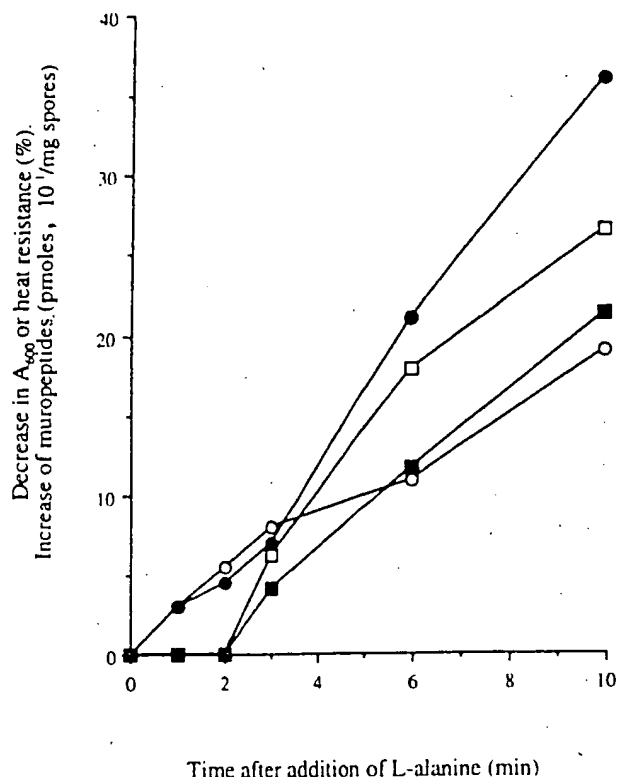


FIG. 4. Kinetics of biochemical events during germination of *B. subtilis* 168 HR spores. ●, percent loss of heat resistance; ○, percent loss of A_{600} ; ■, amount of mucopeptide G3; □, amount of mucopeptide G4.

(25, 28). Modified disaccharide-containing mucopeptides are not apparent, which suggests that the alteration may occur on the δ -lactam moiety. However, the δ -lactams in the modified mucopeptides are still able to be reduced, and acid hydrolysis (2) results in its conversion to muramic acid. Also, 2D NMR spectra did not reveal any alterations in δ -lactam stereochemistry. Similar modifications occur to mucopeptides with tetra- or hexasaccharides and containing either a single L-alanine or tetrapeptide as the side chain, implying that the change occurs close to the muramyl alanine and may be an alteration in stereochemistry. As the modification occurs only on mucopeptides containing the δ -lactam moiety, it is likely that this moiety is required for the activity of the enzyme responsible for the modification. Such requirement for the presence of the δ -lactam moiety for cortex-active enzymes has been previously demonstrated (4, 9). It is possible that epimerase activity can result in a stable alteration in the stereochemistry of the muramic acid residues.

The characteristics of the novel germination exudate-specific mucopeptides match the properties of anhydro-mucopeptides, suggesting the involvement of a lytic transglycosylase in germination (12). This is the first evidence in gram-positive bacteria for lytic transglycosylase activity. There are a number of lytic transglycosylases in *Escherichia coli* which have been characterized at the molecular level (27). The recently released *B. subtilis* genome sequence has revealed the presence of a gene (*yjbJ*) which encodes a putative protein showing high identity (33% over 148 amino acids) to Slt, the major lytic transglycosylase of *E. coli* (7). The possible involvement of YjbJ in germination is currently being investigated.

The anhydro-mucopeptides are almost entirely specific to the germination exudate, although mucopeptide G9 is just detectable in spore-associated material (eluted between mu-

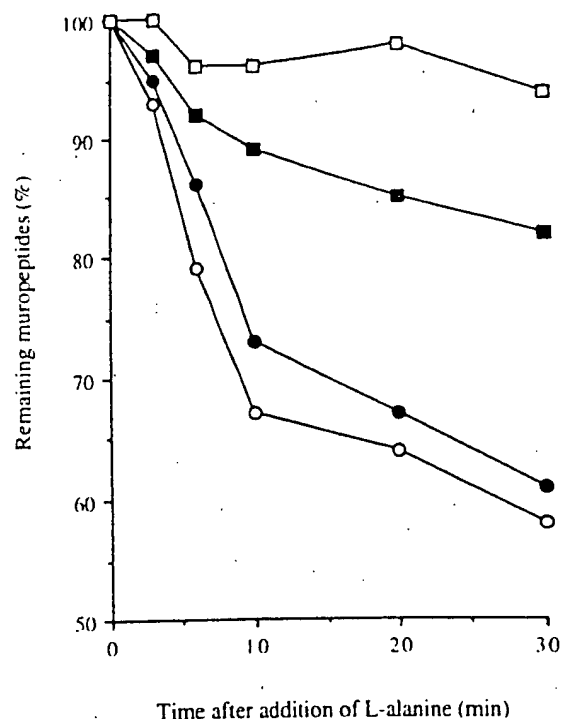


FIG. 5. Differential mucopeptide release during germination of *B. subtilis* 168 HR. Amounts are calculated as a percentage of the dormant spore value. ○, hexasaccharide-containing mucopeptides; ●, tetrasaccharide-containing mucopeptides; ■, disaccharide-containing mucopeptides with alanine or tetrapeptide side chains; □, disaccharide-containing mucopeptides with tripeptide side chains.

ropeptide 12 and G5 [Fig. 1B]). The presence of anhydro-mucopeptides predominantly in the exudate suggests that the lytic transglycosylase acts mostly on released material or at least that which has been previously cleaved by the GSLE (which would result in relaxation of the stress-bearing properties of the polymer). In *E. coli*, the products of lytic transglycosylase activity are also mostly found as soluble material (15).

The anhydro-mucopeptides represent 18.8% of the total mucopeptides released after Cellosyl digestion, 55% of which were found free as single-unit mucopeptides in the exudate without digestion. The free mucopeptides are likely to have been cleaved from the ends of the glycan strands, and thus the lytic transglycosylase is an exoenzyme, processively hydrolyzing the peptidoglycan. Anhydro-mucopeptides represent 60 to 80% of cell wall degradation products released from *E. coli* during autolysis triggered by cephaloridine or trichloroacetic acid (17). In *E. coli*, anhydro-mucopeptides are involved in peptidoglycan recycling and gene regulation (14, 15, 16). The cortex material released during germination is likely to be recycled during the biosynthesis of new peptidoglycan in outgrowing cells (31). Thus, the anhydro-mucopeptides may be recycled and/or form part of a signalling mechanism to initiate new peptidoglycan biosynthesis. We are currently investigating the fate of the germination exudate mucopeptides during spore outgrowth.

The dormancy-maintaining function of the cortex could be relieved solely by the action of the lytic transglycosylase. However, its products are not found in significant levels associated with the germinated spores. It has been suggested that GSLEs may be amidases whose activity would lead to depolymerization of the cortex (10, 23). The remarkably low cross-linking of the spore cortex peptidoglycan (2.9% per muramic acid) would facilitate this process (2). Our study does not reveal direct

evidence for amidase activity during germination in the form of amidase products. However, although the amount of cross-linked cortex material decreases during germination (70% of tetrasaccharide tetrapeptide tetrasaccharide tetrapeptide [muropeptide 19] is lost over 2 h), very low amounts are released in the germination exudate. Therefore, it is possible that amidase activity is occurring. The appearance of trisaccharide tetrapeptide suggests the activity of an *N*-acetylglucosaminidase during germination, although at a very low level. Such an activity has been previously shown to be associated with broken spores of *B. subtilis* (33), *B. megaterium* (13), and *B. cereus* (33). Although a germination-associated muramidase from *C. perfringens* has been characterized (4), there is no evidence for such an activity in *B. subtilis*. To determine the true hydrolytic bond specificity of the GSLE(s); it will be necessary to use purified enzyme and to monitor muropeptide changes associated with its activity on decoated, inactivated spores.

From the analysis of the dynamics of cortex structure during germination, it can be seen that cortex muropeptides containing muramic δ -lactam residues are lost from the spores at a higher rate than those without. Thus, the distribution of muropeptides in the cortex is likely to be heterogeneous. It may be that the muramic δ -lactam residue concentration is greatest in the outer regions of the cortex and thus hydrolysis would be initiated from this area, as the GSLE requires δ -lactam for its activity.

Muropeptides 1 and 8 are disaccharide tripeptide and disaccharide tripeptide disaccharide tetrapeptide, respectively, and their levels remain fairly constant throughout germination. They have been proposed to be part of the primordial cell wall which remains intact during germination, to become the basis of the new vegetative cell wall during outgrowth (2). It is possible that the primordial cell wall contains single L-alanine or tetrapeptide substitutions, but this has not been demonstrated. The primordial cell wall is more cross-linked (20%) than the cortex (2.9%), but it is the absence of the muramic δ -lactam residues which renders this polymer resistant to hydrolysis by GSLE(s), which cannot hydrolyze peptidoglycan without this determinant (2, 25).

Muropeptide analysis has revealed a hitherto unexpected degree of complexity in the mechanism of cortex hydrolysis during germination of *B. subtilis* endospores. We are currently studying structural dynamics during germination of endospores of other species to determine if the mechanism is generic. Identification of the enzymes responsible for the observed activities will allow their role, and how they are regulated as part of the germination trigger mechanism, to be determined.

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Mutations in the *gerP* Locus of *Bacillus subtilis* and *Bacillus cereus* Affect Access of Germinants to Their Targets in Spores

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The *gerP1* transposon insertion mutation of *Bacillus cereus* is responsible for a defect in the germination response of spores to both L-alanine and inosine. The mutant is blocked at an early stage, before loss of heat resistance or release of dipicolinate, and the efficiency of colony formation on nutrient agar from spores is reduced fivefold. The protein profiles of alkaline-extracted spore coats and the spore cortex composition are unchanged in the mutant. Permeabilization of *gerP* mutant spores by coat extraction procedures removes the block in early stages of germination, although a consequence of the permeabilization procedure in both wild type and mutant is that late germination events are not complete. The complete hexacistronic operon that includes the site of insertion has been cloned and sequenced. Four small proteins encoded by the operon (*GerPA*, *GerPD*, *GerPB*, and *GerPF*) are related in sequence. A homologous operon (*yisH-yisC*) can be found in the *Bacillus subtilis* genome sequence; null mutations in *yisD* and *yisF*, constructed by integrational inactivation, result in a mutant phenotype similar to that seen in *B. cereus*, though somewhat less extreme and equally repairable by spore permeabilization. Normal rates of germination, as estimated by loss of heat resistance, are also restored to a *gerP* mutant by the introduction of a *cotE* mutation, which renders the spore coats permeable to lysozyme. The *B. subtilis* operon is expressed solely during sporulation, and is sigma K-inducible. We hypothesize that the *GerP* proteins are important as morphogenetic or structural components of the *Bacillus* spore, with a role in the establishment of normal spore coat structure and/or permeability, and that failure to synthesize these proteins during spore formation limits the opportunity for small hydrophilic organic molecules, like alanine or inosine, to gain access to their normal target, the germination receptor, in the spore.

Spore germination is initiated by the interaction of the germinant molecule with a receptor in the spore. The nature of this receptor is not yet proven, but the available evidence suggests that the genes of the *gerA* family whose products are required for the response to specific germinants are likely to encode this receptor (16, 20). The trigger reaction commits spores to undergo a series of successive events which result in the loss of spore dormancy and resistance properties. Spores of *Bacillus cereus* initiate germination in response to L-alanine or ribosides, of which inosine is the most effective (8). Inhibition of the alanine racemase activity associated with spores by O-carbamyl D-serine is necessary to observe maximum rates of L-alanine-triggered germination, as D-alanine is a competitive inhibitor (8). The first measurable event after commitment is the loss of heat resistance (a rise in spore internal pH, a release of monovalent ions, and a release of dipicolinic acid (DPA) and calcium ions from spores are also early events), and later events include the activation of spore lytic enzymes (7, 17), selective cortex hydrolysis, and rehydration of the spore core. The genetic analysis of spore germination has concentrated on *Bacillus subtilis*; in addition to operons required for germinant-specific responses, such as *gerA*, *gerB*, and *gerK*, genes whose products are required for germination in several germinants have been identified, such as *gerD*. The products of genes

required for the germination response to multiple types of germinant could represent proteins activated by the initial signal transduction mechanism (14). Analysis of *B. cereus* germination mutants has identified germinant-specific loci, such as *gerI*, a homologue of the *gerA* family of operons, required for inosine germination (5). In an attempt to isolate mutants with germination defects in both inosine and alanine, an operon has been identified which, rather than encoding a common element in the germination mechanism, appears to be required for the establishment of spore permeability properties.

MATERIALS AND METHODS

Strains and culture conditions. Strains used in this study are listed in Table 1. Routine culture media were L broth for *Escherichia coli* and Oxoid nutrient broth for *B. cereus* and *B. subtilis*. Synchronous sporulation was by the resuspension method (25). Conditions for spore formation and washing and germination monitoring by loss of optical density (OD) and release of DPA were as previously described for *B. cereus* (5). Spores of *B. subtilis* were prepared and washed as previously described in reference 5, but germination conditions were as described in reference 15, except that the germination buffer was 10 mM Tris-HCl, pH 8.4, containing 2.24 mg of KCl ml⁻¹.

Transposon mutagenesis and mutant screening. Transposon mutagenesis using pLTV1 was as described by Clements and Moir (5). The method of scoring potential germination mutants, modified from that of Irie et al. (10), involved transfer of spore-containing colonies to filter paper and thence onto agar containing specific germinants and 2,3,5-triphenyl tetrazolium chloride, described in detail in reference 5.

DNA sequencing. The sequence of *B. cereus* clones was determined by cycle sequencing by using an ABI 373A DNA sequencer. Sequences were obtained on both strands and were fully overlapped. Staden programs (24) were used for sequence assembly and analysis.

Construction of null mutations in *B. subtilis* genes. Integrational mutagenesis of *B. subtilis* genes with pMUTIN4 (27) used primers internal to the affected genes. For *yisD*, which has 399 bases in the reading frame, bases 34 to 51 and 228 to 245 were used for forward and reverse primers, respectively, with *HindIII*- and *BamHI*-bearing extensions, respectively, to allow cloning into pMUTIN4. For

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Strains		
<i>B. cereus</i>		
569 UM20.1	<i>trp-1</i> Str ^r (wild type for this study)	1, 5
AM1334	Tn917-LTV1::gerPC1	Transposon mutagenesis of UM20.1
<i>B. subtilis</i>		
1604	<i>trpC2</i> (laboratory wild type)	15
BFS3015	<i>yisF</i> ::pMUTIN4 <i>trpC2</i> ; <i>lacZ</i> fusion to <i>yisF</i>	This study
BFS3037	<i>yisD</i> ::pMUTIN4 <i>trpC2</i> ; <i>lacZ</i> fusion to <i>yisD</i>	This study
AM1401	pMUTIN4::yisF <i>trpC2</i>	1604 × DNA (BFS3015) ^a
AM1402	pMUTIN4::yisD <i>trpC2</i>	1604 × DNA (BFS3037) ^a
AM1398	<i>trpC2</i> (parent of BFS strains)	
SH132	<i>trpC2</i> sigKΔ19::pVO12(Pspac-sigK) Cm ^r	19
AM1394	<i>trpC2</i> sigKΔ19::pVO12(Pspac-sigK) Cm ^r <i>yisF</i> ::pMUTIN4	AM1401 × DNA(SH132) ^a
AH64	<i>trpC2</i> metC3 ΔcotE::cat	A. Henriques
AM1423	pMUTIN4::yisF <i>trpC2</i> ΔcotE::cat	AM1401 × DNA (AH64) ^a
Plasmids		
pRS11	Pspac-sigF Cm ^r	R. Schmidt (22)
pDG180	Pspac-sigE Km ^r	21
pDG298	Pspac-sigG Km ^r	26

^a Transformation cross showing the donor strain as DNA.

yisF (615 bases in coding sequence), primers extended from 123 to 141 (forward, with 5' HindIII extension) and 386 to 404 (reverse, with 5' BamHI extension). As discussed in reference 27, these constructions inactivate the gene and create a transcriptional fusion of the gene with a promoterless *lacZ* gene, allowing analysis of expression. A Pspac promoter is introduced downstream of the plasmid insertion.

Permeabilization procedures. Permeabilization procedures for *B. cereus* were based on the UDS method of Brown et al. (3); spores (2 to 4 mg of dry weight ml⁻¹) were incubated at 37°C for 90 min in 5 mM 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) buffer, pH 8.6, containing 8 M urea, 70 mM dithiothreitol, and 1% (wt/vol) sodium dodecyl sulfate (SDS). The spores were then pelleted and washed five times with ice-cold distilled water. Spores were then examined by phase-contrast microscopy to confirm that they remained phase bright, and the permeability to lysozyme was checked by measuring the loss of OD at 580 nm (OD₅₈₀) of an aliquot of the spore suspension incubated in NaCl (50 mM) with lysozyme (30 μg ml⁻¹). This gave 30 to 40% OD loss in less than 30 min, demonstrating that the extraction had removed coat layers sufficiently to allow this enzyme to penetrate to the cortex and induce cortex lysis. The permeabilized spores were heat activated for 30 min at 70°C, then cooled and used within 2 h.

Permeabilization of *B. subtilis* spores (A. Atrih, personal communication) was in 10 mM Tris HCl, pH 8.5, 0.1 M NaCl, 0.1 M dithiothreitol, and 0.5% (wt/vol) SDS. Spore washing, confirmation of permeabilization by lysozyme, and heat activation were all as described for *B. cereus*.

Spore coat extraction procedures. Spore coat extraction procedures with detergents or alkali were as described by Nicholson and Setlow (18). The assay of β-galactosidase during sporulation, using methylumbelliferyl-β-D-galactoside as substrate, was also as described by Nicholson and Setlow.

RESULTS

Isolation of the gerP1 mutant of *B. cereus*. Pools of *B. cereus* 569 UM20.1 cells carrying a chromosomal copy of Tn917-LTV1 were generated as described by Clements and Moir (5), and washed spore suspensions were prepared then enriched for mutants that remained chloroform resistant after incubation in a germinant mixture of alanine and inosine. To increase the proportion of potential germination mutants amongst the survivors, the enrichment procedure was repeated. A colony transfer method of scoring the reduction of tetrazolium chloride by germinating spores was used as a primary screen for germination mutants, by using separate plates with alanine and inosine as germinants. Germination mutants that were strongly germination defective in both alanine and inosine by this test were obtained from all 10 pools of mutagenized spores. How-

ever, there was a likelihood that some of the mutants could contain separate transposon insertions and independent point mutations in separate *ger* genes, selected during the cycling and enrichment procedures. Generalized transduction mediated by phage CP51ts, using a Trp⁺ *B. cereus* 569 strain as recipient, was used to test the linkage of the germination defect to the erythromycin and lincomycin resistance of the transposon. Out of 22 potential *ger* mutants tested, only for two, both derived from the same mutagenesis regime, was the complete germination defect 100% linked to the transposon resistance markers. Most strains contained combinations of a point mutation and a transposon mutation, separately affecting germination in alanine or inosine. Later work revealed that the two mutants were probably siblings, as they contained the identical transposon insertion, and therefore only one, strain AM1334, carrying the mutation Tn917 LTV1::gerP1, is described.

Germination behavior of the gerP1 mutant. Suspensions of the parental strain germinate rapidly and synchronously in either L-alanine or inosine. In contrast, spores of AM1334 (*gerP1*) show no significant loss of OD in inosine, and the rate of OD loss in L-alanine is much reduced (Fig. 1A). Early events in germination do not proceed normally; spores lose heat resistance over a much longer timescale than normal (Fig. 1B), and little DPA is released (Fig. 1C). It was noted that the slower germination behavior was reflected in a reduced colony-forming ability of dormant mutant spores on L Agar or nutrient agar (20 to 25% that of the wild type [2]). Heat activation (70°C for 30 min) improved the colony-forming efficiency of both types of spores approximately threefold, so that 40% of wild-type spores (as counted by light microscopy) formed colonies, but the ratio of wild-type to mutant colony-forming ability remained approximately 5:1.

Germination properties of coat-extracted spores of the gerP1 mutant. Washed permeabilized spores were heat activated, washed once with ice-cold water, and stored on ice. Their germination responses are presented in Fig. 2. Although loss of heat resistance in response to germinant is rapid, permeabilized spore suspensions of the wild type lose much less OD than normal, suggesting that some late germination event has been inhibited by the detergent extraction. However, the

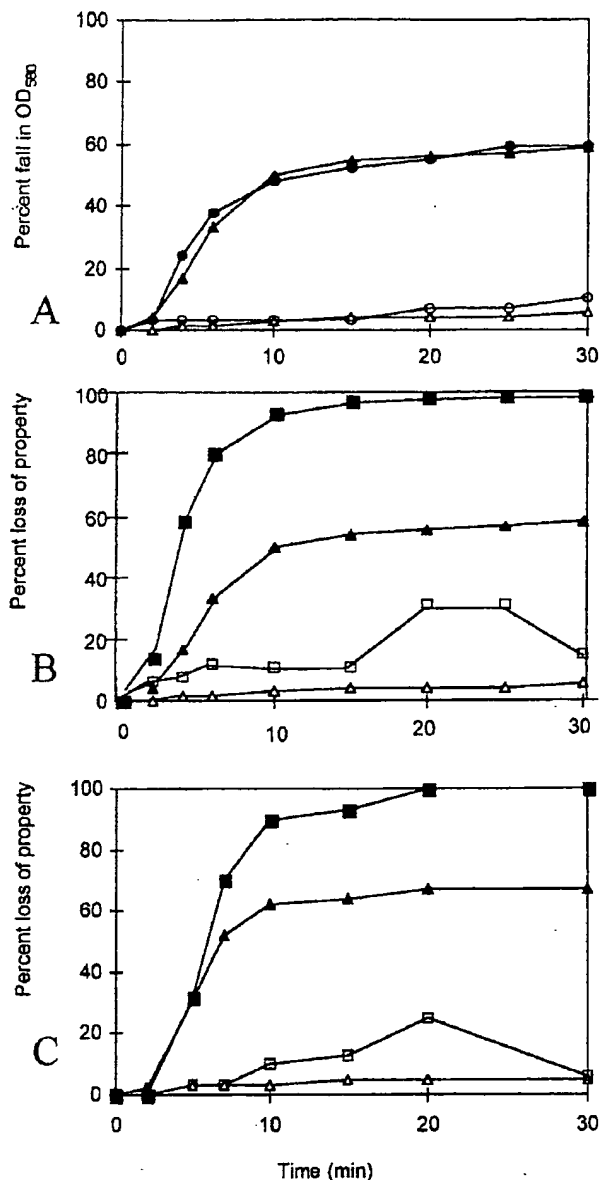


FIG. 1. The germination properties of spore suspensions of *B. cereus* 569 (wild type) and AM1334 (*gerP1*). (A) The fall in OD_{580} of *B. cereus* spore suspensions (wild type) in either L-alanine (●) or inosine (▲) and of *gerP1* in L-alanine (○) or inosine (△). (B) The loss of heat resistance (■) and fall in OD_{580} (▲) of spore suspensions (wild type) and loss of heat resistance (□) and OD loss (△) of *gerP1* in inosine. (C) The release of DPA (■) and fall in OD_{580} (▲) of the wild type and release of DPA (□) and fall in OD_{580} (△) of *gerP1* spore suspensions, germinating in inosine. Panels A, B, and C represent separate experiments.

mutant now responds to germinant in precisely the same manner as the wild type. This demonstrates that the components required for specific germinant-induced early events are still intact in both wild-type and mutant spores. The colony-forming efficiencies of dormant wild-type and mutant spores, after permeabilization, were now identical, at 10^8 per OD unit.

Structure and resistance of *gerP1* spores. The retention of an active germination system in the *gerP1* mutant seen in permeabilized spores suggests that the defect results from a failure

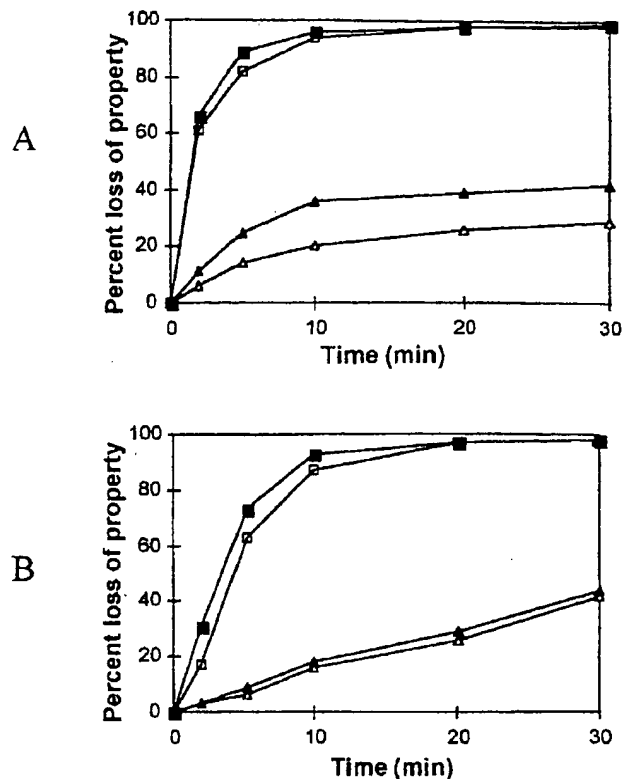


FIG. 2. The germination properties of permeabilized spores of *B. cereus* 569 in inosine (A) or L-alanine (B). Loss of heat resistance (■) and fall in OD_{580} (▲) for spore suspensions of the wild type. The open symbols represent the percent loss of respective properties for the *gerP1* mutant spores.

of the germinant to gain access to its normal target, rather than from an absence of an essential germination component. The properties of mutant spores were therefore examined. Transmission electron microscopy of thin spore sections revealed no detectable difference between the wild type and the mutant (data not shown), and the profile of SDS-polyacrylamide gel electrophoresis-separated coat proteins extracted using detergent or NaOH was unchanged (2); spores of the mutant were lysozyme resistant (2). The colony-forming ability of spores of the wild type and AM1334 after heating in water at 80°C and at 95°C is shown in Fig. 3A and B, respectively. The wild type showed an almost constant logarithmic destruction during heating. The unusual plating behavior of the mutant results in a low recovery of unheated (zero time) spores. The heating of mutant spores resulted in biphasic destruction curves, including an initial increase of recovery, presumably due to activation of the spores, followed by a later logarithmic reduction, which closely matched the inactivation kinetics of wild-type spores. Therefore, an initial activation of the "super-dormant" mutant population appears to be superimposed on a thermal denaturation profile indistinguishable from that of the wild type. The spore cortices of AM1334 and the wild type appeared identical in high-pressure liquid chromatography analysis of digestion products of the cortices (A. Atrih, personal communication). Therefore, no gross defect in either cortex or coat could be detected.

Characterization of the *gerP* locus. Tn917-LTV1 has been designed to allow the rapid cloning in *E. coli* of DNA flanking the site of insertion, as it contains ColE1 replication functions,

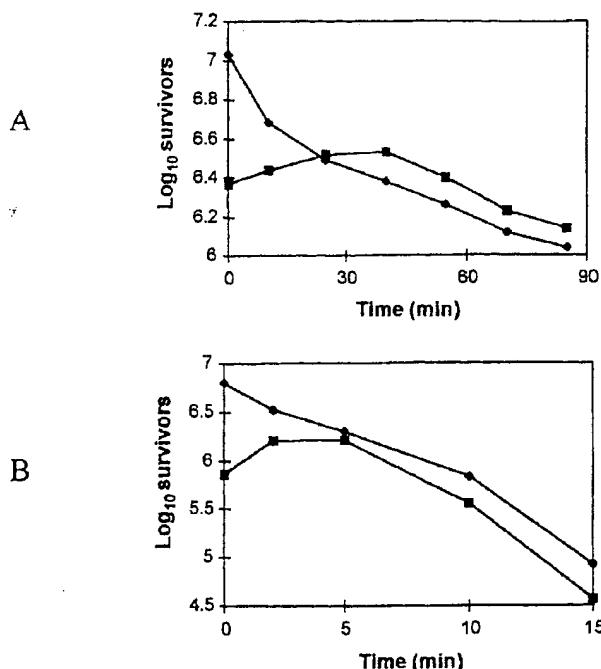


FIG. 3. Isothermal destruction curves for spore suspensions at 80°C (A) and at 95°C (B). Symbols: ♦, wild-type spores; ■, *gerPC1* spores. Suspensions of wild-type and mutant spores were adjusted to the same initial OD.

an antibiotic resistance gene selectable in *E. coli*, and a cluster of restriction sites (4). Chromosomal DNA was isolated from AM1334, digested with *EcoRI*, diluted, ligated, and then used to transform *E. coli* DH5 α . The plasmid recovered (pJBD1) contained the expected vector fragment and a 1.8-kb insert.

Only DNA from the *lacZ*-proximal side of the transposon is recovered by this means. A λ ZAP Express (Stratagene) library of *B. cereus* chromosomal DNA containing fragments of 4 to 9 kb from a partial *Sau3A* digest was constructed and probed with the insert fragment from pJBD1. Two hybridizing phages were purified, and phagemids pJB1 and pJB2 were excised. The larger, pJB1, contained a 5.5-kb insert, encompassing the complete *gerP* region (Fig. 4). The sequence of the cloned region on either side of the point of transposon insertion has been determined and deposited in GenBank (accession no. AF053927). This revealed a cluster of six genes (*gerPA* to *gerPF*) followed by a potential rho-independent terminator (Fig. 4). The putative operon is preceded by a small gene (named *yisI*, to correspond with its *B. subtilis* homologue, as discussed below). This gene would be transcribed in the same direction as *gerP*, but is separated from the *gerP* region by a potential rho-independent terminator. Potential ribosome binding sites (RBSs) are appropriately located for each open reading frame (ORF). Those for *gerPB*, *gerPD*, and *gerPE* all overlap with the end of the previous ORF; in contrast, there are two longer intergenic regions: a 52-base region between the stop codon of *gerPB* and the RBS of *gerPC*, which contains the site of transposon insertion in the *gerP1* mutant, and a 26-base region between the stop codon of *gerPE* and the RBS of *gerPF*. The organization and relationships between gene products described below suggests that the six ORFs are likely to represent an operon. Another ORF on the *gerP*-distal side of *yisI*, and read in the opposite direction, was partially sequenced and was found to be a homologue of *yisK* of *B. subtilis* (2).

Four of the GerP proteins are relatively small (64 to 73 residues), and all except GerPB have a predicted pI in the acidic range. The only region of hydrophobic amino acid sequence long enough to represent a membrane-spanning helix is found at the N terminus of GerPE. The GerPA, GerPB, GerPD, and GerPF proteins are related in sequence, with, for example, 42% identity between GerPA and GerPF (Fig. 5). GerPB and GerPD are related, and their N-terminal half

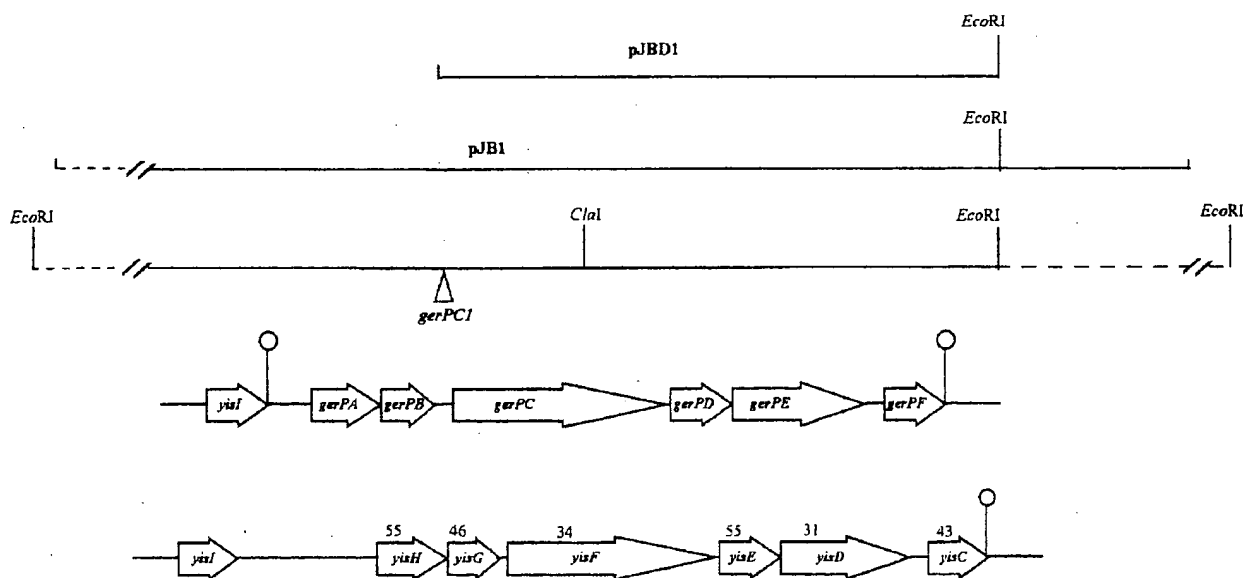


FIG. 4. The gene organization of the *gerP* locus in *B. cereus* and *B. subtilis*. The extent of *B. cereus* clones is indicated above the chromosomal region, which shows the point of transposon insertion. The *B. cereus* *gerP* operon and the homologous *B. subtilis* operon (*yisH* to *yisC*) are shown. Figures above the *B. subtilis* ORFs indicate percentage of amino acid identity to the equivalent *B. cereus* ORF.

YisH	1	MPALVGAFKINAIGTSCVYHIGDCITISPPQAQVRTFAGAGSFHTGDBLKVMNY
GerPA	1	MPAMVGHIRIVNIGSSGIEHIGDVFAIRPISYSRATFAGACSFHVGDNVSVINY
YisC	1	MSPMEATVGPIAINSLSGGVNFGDFYLSPKSSSKSALGSGAGHTGDFLLNNA
GerPF	1	MPBVGNLVVQN..SNGSFNLGDFYNVSEKENTKAYNGSSASNVGFVNTFNG
YisE	1	MIFTVIHRSLEVGDIRMHGVS	SSSVFHHIGDTEH.YLSSIFDTPPESLIIGFPAPLAF.
GerPD	1	MNLNVVRELKVGQIKMHGVS	SSSALEFLIGDANLL.ILSSILDTPPFTVTEGPFVPLVTDV
YisG	1	MNF.YINQTIQINYLRLSISNS	SILOIGSAGSIKSLSNLYNTGSYVEPAPEVSGSQPL
GerPB	1	MNF.YVNRQSIINSTIKIDSITTS	SVFQIGTAGSIKALSXPFSNTGGEFTEPLRPLQAKQII

YisH	54	QNATNVY	DNDAVDQPIVANA
GerPA	54	QSATTVNDSDVDQAIIGST	
YisC	56	VNATNYIDEDVNDQDMVGNG	
GerPF	52	VSAATNTFDSVDADQDQIGTA	
YisE		
GerPD	60	PPTPG	
YisG	60	QLQEPDTGSLVPLQPPGR..	
GerPB	60	SIKPSTSS

FIG. 5. An alignment of the primary sequences of homologous protein products of the *gerP* locus of both *B. cereus* and *B. subtilis*. The equivalent genes in the two species are adjacent in the alignment.

shares homology with that of the GerPA-GerPF pair. The C-terminal half of GerPB-GerPD is less conserved, and it is rich in glycine, proline, and alanine residues, suggesting an extended structure. The GerPC protein, at 204 amino acids, and GerPE, at 128 residues, are encoded by the larger ORFs and have no homologues.

An operon homologous to *gerP* is present in the *B. subtilis* genome sequence (13) (Fig. 4). The gene organization in the immediately surrounding region is identical in the two species, except that the flanking *B. subtilis* *yisJ* and *yisB* genes have no counterpart in this region in *B. cereus*. The degree of amino acid identity for the ORFs of the *gerP* operons of *B. subtilis* and *B. cereus* is indicated in Fig. 4 above the *B. subtilis* ORFs.

Mutation of the *yisH-yisC* (*gerP*) operon of *B. subtilis*. The germination properties of spores of *yisF* and *yisD* mutants of *B. subtilis*, generated by integrational inactivation with pMUTIN4 (27), and transfer of the mutations into our laboratory strain, are compared with the wild-type parent in Fig. 6. Both mutants germinate slowly in alanine, and also slowly in the alternative combination of germinants for *B. subtilis*, asparagine, glucose, fructose, and KCl. The rate of OD loss is higher than that seen for the *B. cereus* mutant; the defect is less extreme in *B. subtilis*. This behavior is matched by the normal plating efficiency of these mutants in *B. subtilis*. Chemical permeabilization of the spores increased the germination rate in response to both germinants (Fig. 7 shows the data for germination in L-alanine). The permeabilization conditions used for *B. subtilis* were less harsh, and only 60% of the *yisF* mutant spores had been permeabilized to lysozyme (compared to >90% for the wild-type, *yisD* mutant, and *B. cereus* spores). This probably explains the lower response of the *yisF* mutant spores after chemical permeabilization compared to the other preparations.

In *B. subtilis*, the disruption of function of either the *yisF* or *yisD* gene causes a generally similar, though less extreme, defect in spore physiology to that observed in the *gerPC* transposon mutant of *B. cereus*, in which expression of the last four ORFs of the equivalent operon is disrupted by the transposon insertion. It is not known whether the less extreme defect introduced by the *yisD* and *yisF* mutations in *B. subtilis* reflects difference in the importance of these proteins in the *B. subtilis* spore coat or whether it results from residual function of the intact upstream genes (or downstream genes, as an uninduced *Pspac* promoter is present) in the *gerP* operon in these insertional mutants.

Consequences of introduction of a *cotE* mutation. A *cotE* *yisF* mutant was constructed to test whether permeabilization

of the coats by introduction of a *cotE* mutation (6) gave the same result as chemical permeabilization. The lysozyme sensitivity of spores of the double mutant was confirmed, and washed spores were germinated in L-alanine. Germination, as estimated by loss of heat resistance of spores, was increased twofold on introduction of the *cotE* mutation, restoring the germination response to that of the wild type (Fig. 8).

Regulation of expression of the *yisH-yisC* (*gerP*) genes of *B. subtilis*. Although the transposon used for *B. cereus* mutagenesis carried a *lacZ* reporter, the insertion in *B. cereus* AM1334

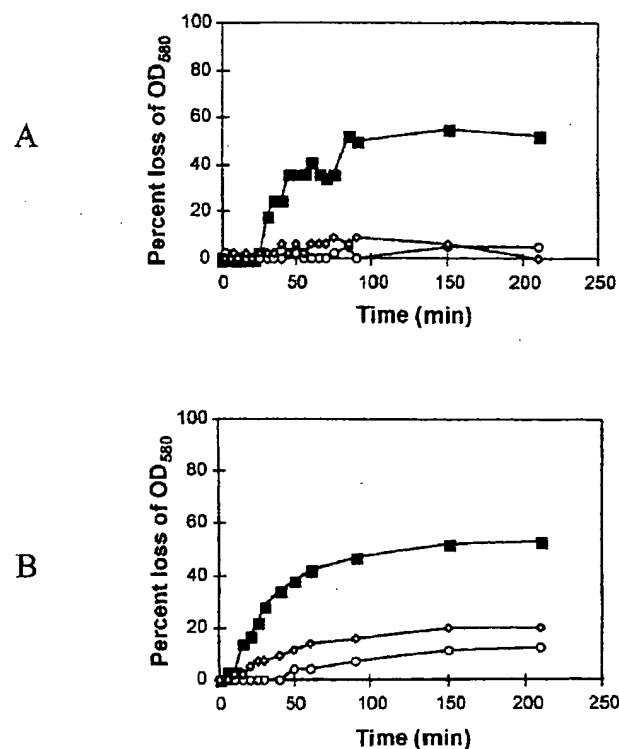


FIG. 6. Fall in OD₅₈₀ of *B. subtilis* spore suspensions in 1 mM L-alanine and 10 mM KCl (A) or AGFK (20 mM asparagine, 8 mM glucose, 8 mM fructose, and 20 mM KCl) (B). Symbols: ■, 1604 wild type; ◇, AM1402 *yisD*; ○, AM1401 *yisF*.

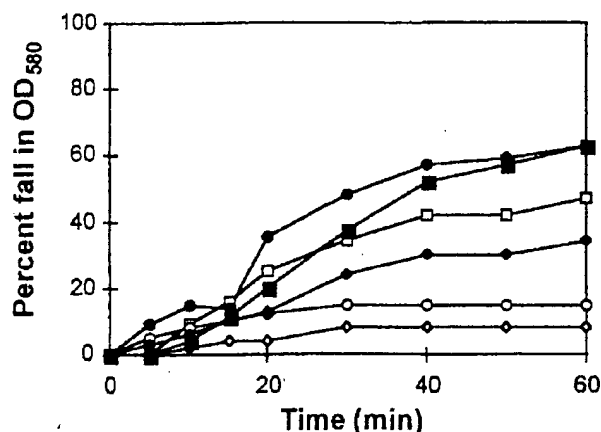


FIG. 7. The fall in OD_{580} of *B. subtilis* spore suspensions after permeabilization. Symbols for different spore suspensions are \square , wild-type spores; \circ , *yisD* (AM1402); and \diamond , *yisF* (AM1401) mutant. Open symbols represent intact, non-permeabilized spores. Solid symbols represent the fall in OD_{580} of the respective suspensions of permeabilized spores.

(*gerPC1*) was in the wrong orientation to create a transcriptional fusion to the *lacZ* gene. The insertional mutagenesis in *B. subtilis* was designed specifically to create such fusions, and measurement of *lacZ* expression in AM1401 and AM1402 cultures induced to sporulate synchronously (25), by using a sensitive fluorescence assay, reveals that expression of *yisF* and *yisD* is switched on at the same time, after 3 h of sporulation (Fig. 9). This level of expression is just detectable using a classical *o*-nitrophenyl- β -D-galactopyranoside assay, but is easily measured with the fluorogenic substrate methylumbelliferyl- β -D-galactoside.

The spatial and temporal control of gene expression during sporulation is mediated by successive sporulation-specific sigma factors. The introduction of isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible versions of these sporulation sigma factors, under *Pspac* control, resulted in expression of *yisD* on induction of sigma K, but not on induction of sigmas E, F, or G. (Fig. 10). Similar results were obtained for *yisF* (data not shown). Examination of the sequence upstream of the first gene of the cluster in both species reveals sequences that could

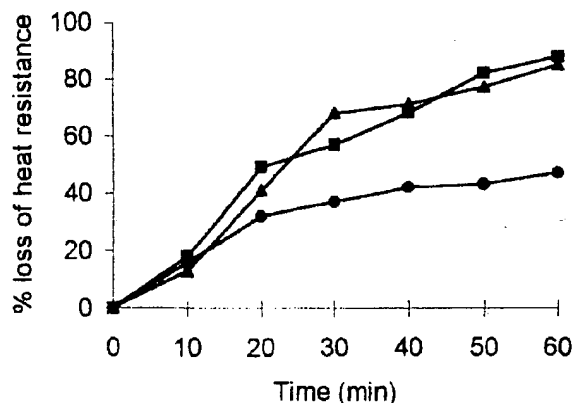


FIG. 8. Germination of *B. subtilis* spores in L-alanine, as measured by loss of heat resistance (70°C for 30 min). Squares represent 1604 (wild type), circles represent AM1401 (*yisF*), and triangles represent AM1423 (*yisF cotE*).

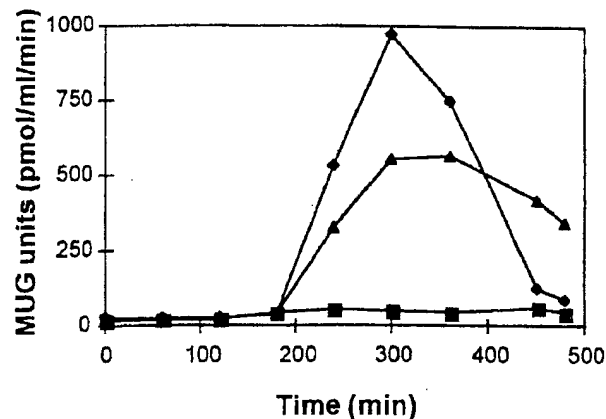


FIG. 9. Expression of *lacZ* fusions to *yisD* (\blacktriangle) and *yisF* (\blacklozenge) during synchronous sporulation. \blacksquare , the β -galactosidase activity of a control strain (1604). MUG, methylumbelliferyl- β -galactoside.

represent potential sigma K-dependent promoters. Introduction of the *yisD-lacZ* or *yisF-lacZ* fusions into a *gerE36* mutant background resulted in dramatic overexpression of these genes, as estimated by a plate assay, spraying the fluorogenic substrate on sporulating colonies. A more detailed analysis would be necessary to determine whether this reflects a direct role of GerE in negative regulation of these genes, or possibly an indirect effect, resulting from the increased levels of sigma K in a *gerE* mutant (9).

DISCUSSION

This work has identified a novel cluster of genes, organized in an operon-like arrangement, that is required for formation of functionally normal spores in both *B. cereus* and *B. subtilis*. These genes are only expressed during sporulation, in the mother cell compartment around the time of spore coat synthesis and assembly. Expression is sigma K-dependent and

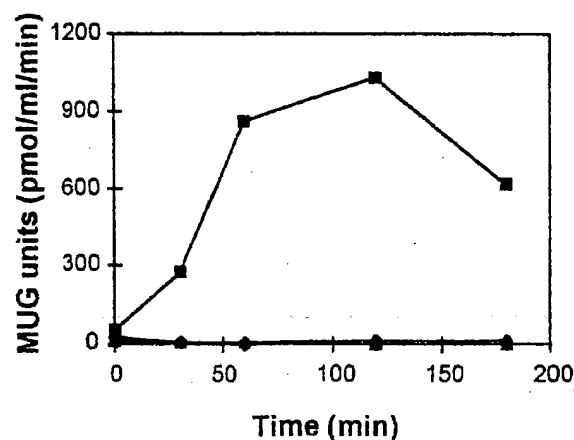


FIG. 10. The expression of a *yisD-lacZ* fusion by induction of sporulation-specific sigma factors during vegetative growth. Symbols: \blacklozenge , sigma F; \bullet , sigma E; \blacktriangle , sigma G; and \blacksquare , sigma K. With the exception of the sigma K-inducible strain (AM1394), the sigma factors were carried on the relevant plasmids described in Table 1, introduced into AM1402. Graphs for sigma F, sigma E, and sigma G induction are superimposed on the baseline—no *lacZ* induction was observed.

negatively regulated by GerE, a major regulator of coat protein gene expression (6). The proteins may be structural components of the spore or components required during the morphogenetic process but not represented in the mature spore. Immunochemical analysis would be required to distinguish these possibilities. The absence of some of these proteins does not appear to result in any major changes in spore structure, as revealed by transmission electron microscopy, or any major changes in coat protein composition, as demonstrated by gel electrophoresis of extracted proteins. The expression of the gene cluster in *B. subtilis* is easily detectable by *lac* fusion analysis, but the level of β -galactosidase synthesis is not as high as would be expected for highly expressed genes encoding major coat proteins.

The outer layers of spore coat and integument in *B. subtilis* and *B. cereus* are rather different in ultrastructure: *B. cereus* spores have a coat that appears thinner, in terms of the number of coat layers, and the spores are surrounded by an exosporium. The range and size of extractable spore coat proteins is also very different. Despite the extensive analysis of coat genes and proteins in *B. subtilis*, there has so far been little study of the molecular composition of integument layers in *B. cereus*. In both types of spores, however, the absence of at least some of the GerP proteins causes a defect in spore germination, more extreme in *B. cereus*, which can be relieved by extraction of coat layers sufficiently to permeabilize the spore to lysozyme. The residual defect in loss of heat resistance in response to germinant in a *B. subtilis* *yisF* mutant is overcome on introduction of a *cotE* mutation, which causes a defect in assembly of the spore outer coat and an increase in spore permeability (6).

The effect of coat protein extraction on germination of *B. cereus* T spores in a mixture of alanine and inosine has already been described (12, 23). Extraction does not inhibit the response of the spore to germinants as determined by loss of heat resistance, although it does reduce the amount of OD loss observed. Germination by inosine and alanine is dependent on more than one class of GerA homologues (5), but the response to each of these individually in the *gerP* mutant is similarly affected. As the germinant-specific response is still observed, the primary initiating target for germinants in the spore is unaffected by this extraction of outer layer proteins, although the completion of later events is severely disturbed. The intact spores of the *B. cereus* *gerP* mutant represent a type of superdormant spore, whose latency can be overcome to some extent by extreme heating or by extraction of the spore, permeabilizing it to molecules of the size of lysozyme. It appears that the integument in the *gerP* mutant may be abnormally impermeable to germinants, as on its removal, they can once more access their primary target(s) with at least the normal kinetics. The inner coat layers may represent a general barrier to the passage of small organic molecules, as reported for glucose at 4°C in *Bacillus megaterium* QMB1551 (11).

The GerP proteins could contribute directly to a structural element normally present in the spore that facilitates transfer of such molecules across the integument under physiological conditions; an alternative interpretation is that they are required for proper assembly of other coat proteins into a structure that allows passage of germinants. Whichever is the correct interpretation, the phenotype of the *gerP* mutants focuses attention on our lack of understanding of the permeability properties of the outer layers of the bacterial spore and on the need of germinant to traverse these layers to initiate the early stages of the germination response.

To give these genes a designation based on the germination

defect of mutants is not ideal, but this at least indicates an associated phenotype. In the absence of evidence of a direct coat defect, we have adopted the *gerP* terminology for *B. cereus* and suggest that the same gene designations be adopted in *B. subtilis*.

ACKNOWLEDGMENTS

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medium (Monod and Jacobson). This indicates that the regulating gene, *i*, can reduce or β -galactosidase synthesis, as that of an *R1a*⁻ mutation on atase synthesis.

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PERMEABILITY OF BACTERIAL SPORES

III. PERMEATION RELATIVE TO GERMINATION¹

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ABSTRACT

BLACK, S. H. (The University of Michigan, Ann Arbor) AND PHILIPP GERHARDT. Permeability of bacterial spores. III. Permeation relative to germination. *J. Bacteriol.* 83:301-308. 1962.—The passive diffusion of solutes into dormant spores, characterized previously with the test organism *Bacillus cereus* strain terminalis, has now been examined in relation to germination. Dormant spores did not take up specific germinants differently than they did other compounds, under conditions optimal for germination. Germinated spores, viable but prevented from growing out, displayed some changes in permeability, evidenced by increased total uptake of glucose and water and by observable penetration of a fluoresceinogenic dye. Heat-killed spores were as permeable to glucose and the dye as germinated ones.

How dormancy in bacterial spores is maintained, and then on chemical or physical signal broken, has long been subject to speculation. One view has been that inertness is a result of isolation from the environment—that is, a spore is impermeable. Results of experiments testing this view proved the opposite (Black and Gerhardt, 1961; Gerhardt and Black, 1961). Moreover, the fact that a spore can germinate in an appropriate chemical environment would seem to make permeability indispensable. But does the dormant spore take up specific chemical germinants differently than it does other compounds? Is germination accompanied by a change in permeability? If so, will disruptive treatments that might be expected to simulate the effects of chemical germinants also affect solute uptake? Such points were examined in the experiments reported below.

¹ A preliminary account of this study was presented at the VIIth International Congress for Microbiology, 1958.

MATERIALS AND METHODS

Details of the materials and methods have been described previously (Black and Gerhardt, 1961; Gerhardt and Black, 1961). The test species again was *Bacillus cereus* strain terminalis, for which procedures have been developed to obtain hectogram masses of clean, dormant spores. Although the chemical requirements for germination of these spores were known (Church, 1955), the procedures employed for the usual dilute suspensions, even with proportional increases, did not effect rapid and complete germination when dense suspensions (about 10^{11} spores per ml) were used.

Eventually, the following procedure proved successful: Clean dormant spores in water suspension (approximately 50%, w/v) were heated at 65 C for 2 hr. After the spores were centrifuged and the supernatant water decanted, packs of about 10 g wet weight were resuspended in 50 ml of Trypticase soy broth (Baltimore Biological Laboratories) supplemented with 300 mg of L-alanine and 200 mg of adenosine. These suspensions were placed in 250-ml Erlenmeyer flasks and incubated at 30 C on a rotary shaker for 30 min. Germination of the spores was evidenced by their darkness under the phase microscope, stainability, and heat sensitivity; the completeness of germination was judged from the fact that repeated examination of heavy smears on slides did not reveal any ungerminated spores. The spores were separated from the germination broth by centrifugation and then washed twice with deionized water. During final resuspension of the germinated spores, care was exercised not to disturb the tightly packed pellet of crystalline dipicolinic acid, which was released during germination and collected at the bottom of the centrifuge tube. The spores were distributed in 3-g samples into tubes and centrifuged for 30 min at $17,000 \times g$; after draining, drying, and weighing the tubes, the washed germinated spores were ready for use. The germinated spores pre-

pared in this way were stable, did not develop into vegetative cells, and remained fully viable for about 3 days if refrigerated. The principle has since been extended to arresting stages of outgrowth (Goldman and Blumenthal, 1961).

As before (Black and Gerhardt, 1961), the uptake of exogenous materials by spores was assayed by the space technique: the resulting space value (R^w), which is corrected for interstitial space and is calculated on a spore weight basis, indicates the percentage of the spore itself that is permeated. The antecedent values for S^w represent the pack space; these are included in one of the tables (Table 4) below.

A possible change in permeability with spore germination was also assessed microscopically by observing uptake of the ultraviolet-fluorescing dye, *n*-tolyl- α -naphthylamine-8-sulfonic acid (National Aniline Division, Allied Chemical Corporation). For fluorescence microscopy, a Reichert 'Fluorex' illuminator and a conventional dark-field microscope were employed. Light from a 200-w maximum-pressure mercury-vapor source (Osram HBO-200) in the illuminator was directed through two pass-filters (Schott UG1-2mm and UG1-1mm) with an optimal passage at 365 $m\mu$. Contact between the microscope condenser and the specimen slide was maintained with immersion oil. The ocular contained an excluding filter (Schott GG 9) barring light below 500 $m\mu$. For spores, it was necessary to use a 20 \times objective (0.65 NA) and a 12.5 \times ocular. The observations were recorded on Eastman Tri-X film.

RESULTS

Uptake of germination compounds by dormant spores. Spores of *B. cereus* strain terminalis can germinate with adenosine plus L-alanine or glucose (Church, 1955); the rate of germination increases with prior heating at 65 C for 15 min for dilute suspensions. The space in dormant spores available to glucose has been determined to be about 40% and to be essentially independent of environmental variables, including temperature (Black and Gerhardt, 1961); other sugars, ineffective as germination agents, were taken up by dormant spores to about the same degree (Gerhardt and Black, 1961). L-Alanine also failed to penetrate to an unusually high degree and, in fact, was found to be admitted into dormant spores to a consistently lesser extent (32%) than most small molecules. As shown in Fig. 1, both glucose and alanine could be water-

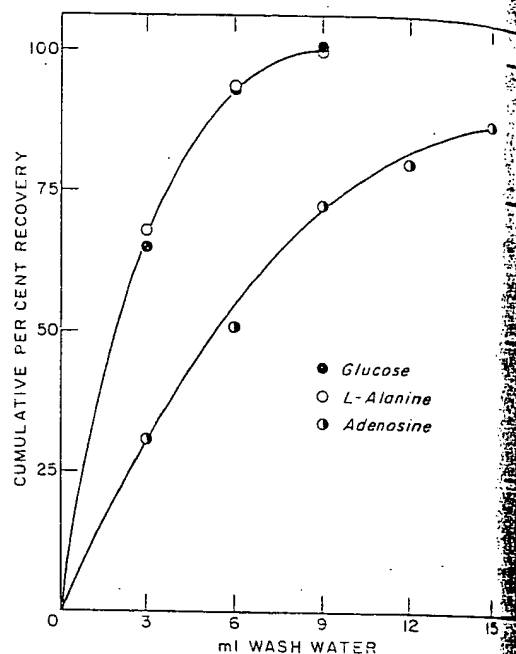
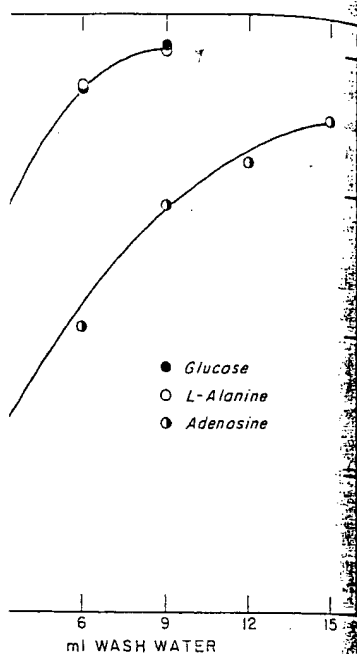


FIG. 1. Elution of germination compounds from dormant spores. Each tube contained about 3 g of wet spores which were washed with 3-ml increments of water.

eluted completely and readily. Both L- and D-alanine were taken up to an equal degree, a finding at variance with the stereospecificity in binding of L-alanine reported by Harrell and Halvorson (1955). However, the amount they reported bound is so small (about 5×10^{-4} moles per spore) that it would be obscured by the predominantly diffusional uptake occurring with the high concentrations used in the present experiments.

In contrast to alanine and glucose, adenosine was taken up by dormant spores to a high degree. As shown by the results in Table 1, however, several lines of evidence suggest that the high but variable adenosine space was attributable to nonspecific adsorption: (i) The amount of adenosine taken up was a function of the quantity of cells available for binding. (ii) The adenosine taken up was resistant to removal by water washing (Fig. 1) unless the pH was reduced near the pK_a (3.45) for this riboside. (iii) The similarly basic adenine component, but not ribose, gave a comparably high uptake value; neither moiety, however, substitutes for adenosine as a germinant.

Although glucose, alanine, or adenosine uptake



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TABLE 1. Evidence for adsorptive uptake of adenosine by dormant spores

Solute	Equil time	H _p	R ^w	Cumulative recovery in two water washes	
				pH	%
Adenosine	15	3.00	76	7.0	35
Adenosine	15	3.49	119	3.0	78
Adenosine	60	3.49	121	7.0	58
Ribose	15	3.19	41	7.0	90
Adenine	15	3.16	112		

by dormant spores was uninfluenced by incubation at a temperature optimal for germination or by mild heating of the spores (Table 2), it seemed possible that the permeation of these compounds might be different if present in combination, as required for germination. Neither glucose nor alanine in the presence of adenosine, nor adenosine in the presence of glucose or alanine, showed a greater uptake than when presented singly to the spore (Table 3).

Uptake by germinated spores. The first definable event in germination is often considered to be a break in the impermeability of the spore. The evidence that has led to this concept, however, is considerable but only circumstantial. Germinating spores lose their refractility and become dark under phase optics (Pulvertaft and Haynes, 1951; Powell, 1957; Knaysi, 1959; Rode and Foster, 1960), decrease in dry weight by about one-third (Powell and Strange, 1953), exude most of their dipicolinic acid and much of their calcium and a nondialyzable mucoprotein into the germination medium (Powell and Strange, 1953), resume active respiration (Murrell, 1955), and become uniformly stainable with basic dyes (Grethe, 1897; Leifson, 1931; Murrell, 1955). Germinating spores also enlarge (Cohn, 1877; Fischer, 1897; Grethe, 1897; Leifson, 1931; Rode and Foster, 1960), suggesting imbibition of water. Direct evidence for water uptake is lacking, but further indirect evidence is provided by the inability of spores to germinate in environments of low water activity, that is, in concentrated solutions of sucrose (Beers, 1957), triethylene glycol, or sodium chloride (Black and Gerhardt, unpublished data).

Permeability determinations on germinated spores are compiled in Table 4. The test compounds were selected from among those used

TABLE 2. Uptake of germination compounds under various conditions of incubating spores

Conditions	R ^w		
	Glucose	L-Alanine	Adenosine
0 C for 15 min	40	32	76
30 C for 15 min	42	31	71
30 C for 15 min after heating spores at 65 C for 30 min	40	33	74

TABLE 3. Uptake of germination compounds, singly and in combination, by dormant spores

Compound measured	Additional compound present	R ^w
Glucose-U-C ¹⁴	None	39
Glucose-U-C ¹⁴	Adenosine	39
Adenosine-S-C ¹⁴	None	78
Adenosine-S-C ¹⁴	Glucose	81
Adenosine-S-C ¹⁴	DL-Alanine	78
DL-Alanine-1-C ¹⁴	None	32
DL-Alanine-1-C ¹⁴	Adenosine	31

previously with dormant spores (Table 1 in Gerhardt and Black, 1961); the same identifying numbers are retained in Table 4, and the R^w values for dormant spores are reproduced in parentheses for convenience. The antecedent S^w values for germinated spores include figures to allow judgment of the reliability and variability of the permeability determinations.

At once evident from the results shown in Table 4 was the greater uptake of water by germinated spores (R^w = 76%) as compared to dormant spores (R^w = 67%). This immediately suggested the possibility that the increased uptake also observed for some small solutes, glucose especially, was associated with the increased water content. Another possibility was that a lipidlike component of spores is lost during germination, since repeated permeability determinations with glucose showed that its uptake was no longer restricted, as for small lipid-insoluble compounds in dormant spores.

Molecular weight principally regulated solute uptake in dormant spores, and a graphical analysis of this factor for germinated spores is presented in Fig. 2. Within the limitations of the number and scatter of points, the graph revealed

TABLE 4. Permeability of germinated spores to selected compounds*

Compound		Experimental conditions			S ^r			R ^r _{avg}
No.	Species	Solute concn	Temp	Equil time	No. of dets	Range	Avg	
		g/100 ml	C	min		%	%	%
113	Water		4	15	10	76-86	78	76 (67)
2	Glucose	1	4	15	10	55-59	56	51 (40)
25	Dextran 3,650	3	4	60	3	32-42	37	30 (28)
27	Dextran 10,000	3	4	60	4	24-32	26	18 (25)
29	Dextran 19,000	3	4	120	1	17	17	7 (12)
31	Dextran 40,000	3	4	60	4	11-20	16	7 (11)
34	Dextran 150,000	3	4	60	4	12-13	12	2 (2)
36	Dextran 500,000	3	4	60	4	6-11	8	-1 (0)
37	Dextran 700,000	3	4	15	33	7-10	9	0 (0)
38	Dextran 2,000,000	3	4	60	3	6-13	10	1 (0)
40	Ethylene glycol	3	4	60	3	67-71	69	64 (53)
42	Triethylene glycol	3	4	60	2	61-69	66	62 (49)
46	Polyethylene glycol 400	3	4	60	3	45-48	47	41 (38)
48	Glycol 1,000	3	4	60	3	28-38	33	26 (35)
50	Glycol 3,350	3	4	60	3	25-32	29	21 (28)
52	Glycol 17,500	3	4	60	3	21-26	24	16 (15)
53	Glycol 70,000	3	4	60	3	19-22	21	12 (4)
71	D-L-Alanine	1	4	15	6	53-58	56	50 (32)

* Numbers of the compounds correspond to those given in Table 1 of Gerhardt and Black (1961). The R^r values in parentheses are for dormant spores, reproduced from the same source.

several trends: (i) An inverse relationship was preserved between uptake and the log of the molecular weight, and the line slope (-17.5) was only slightly steeper than that for dormant spores (-16.4). (ii) The inflexion point in the correlation line was about the same for the two spore types (130,000 mol wt for germinated spores and 160,000 mol wt for dormant spores), indicating that the loss of polymeric constituents during germination did not change the maximal porosity of the spore coat. (iii) The plateau at an S^r value of about 9, which represents the intersporal space and is equivalent to an R^r value of 0, was maintained.

The foregoing results evidenced some changes in permeability, notably to water and glucose, accompanying germination of spores. It also seemed desirable to visualize the changes, if possible. In a sense, one does so with simple stains, but the ease with which germinated spores stain entirely does not distinguish between a change in permeability and a change in affinity. Newton (1954), however, has used a fluorescing dye to demonstrate a change in permeability that occurs in polymyxin-treated bacteria. The dye, *n*-tolyl- α -naphthylamine-8-sulfonic acid

(TNS) fluoresces in ultraviolet light if conjugated with negatively-charged groups of protein. Intact *Pseudomonas aeruginosa* cells in the presence of TNS could not be detected in a fluorimeter, but addition of polymyxin immediately resulted in fluorescence, indicating that TNS penetrated to intracellular proteins. This dye has been used in our laboratory for fluorescence microscope examination of hexachlorophene-treated cells (Joswick and Gerhardt, *unpublished data*), and its use seemed especially applicable to the present problem in spores.

To test whether penetration of TNS occurred, spores were germinated, washed, and placed in an 0.85% (w/v) saline solution of 0.001 M TNS. Germinated spores (Fig. 3A) were seen as distinct, brightly fluorescing points; by contrast, the dormant spores (Fig. 3C) were identifiable only by an ill-defined blur. The dim fluorescence of dormant spores on this 30-min photographic exposure was surprising, since the same field was uniformly dark to the eye and to film exposed only 1 min, a time sufficient to record fluorescence of germinated spores. The picture of dormant spores suggested halation, that is, scattering of light beyond its proper boundary, which might

lected compounds*

S ^w		R ^w , avg
Range	Avg	
%	%	%
76-86	78	76 (67)
55-59	56	51 (40)
32-42	37	30 (28)
24-32	26	18 (25)
17	17	7 (12)
11-20	16	7 (11)
12-13	12	2 (2)
6-11	8	-1 (0)
7-10	9	0 (0)
6-13	10	1 (0)
67-71	69	64 (53)
61-69	66	62 (49)
45-48	47	41 (38)
28-38	33	26 (35)
25-32	29	21 (28)
21-26	24	16 (15)
19-22	21	12 (4)
53-58	56	50 (32)

* 1 c. Gerhardt and Black (1961)
from the same source.

in ultraviolet light if conjugated charged groups of protein. Intact *Trichosporon* cells in the presence of TNS could not be detected in a fluorimeter, but TNS immediately resulted in fluorescence indicating that TNS penetrated to the interior of the cells. This dye has been used in fluorescence microscope examination of chlorophyll-treated cells (Josserand, unpublished data), and its use is especially applicable to the present study.

After penetration of TNS occurred, spores were germinated, washed, and placed in a saline solution of 0.001 M TNS. Spores (Fig. 3A) were seen as discrete fluorescing points; by contrast, spores (Fig. 3C) were identifiable as a diffuse blur. The dim fluorescence observed on this 30-min photographic exposure was surprising, since the same field was exposed to the eye and to film exposed for a sufficient time to record fluorescence from dormant spores. The picture of dormant spores, that is, scattering of light from a proper boundary, which might

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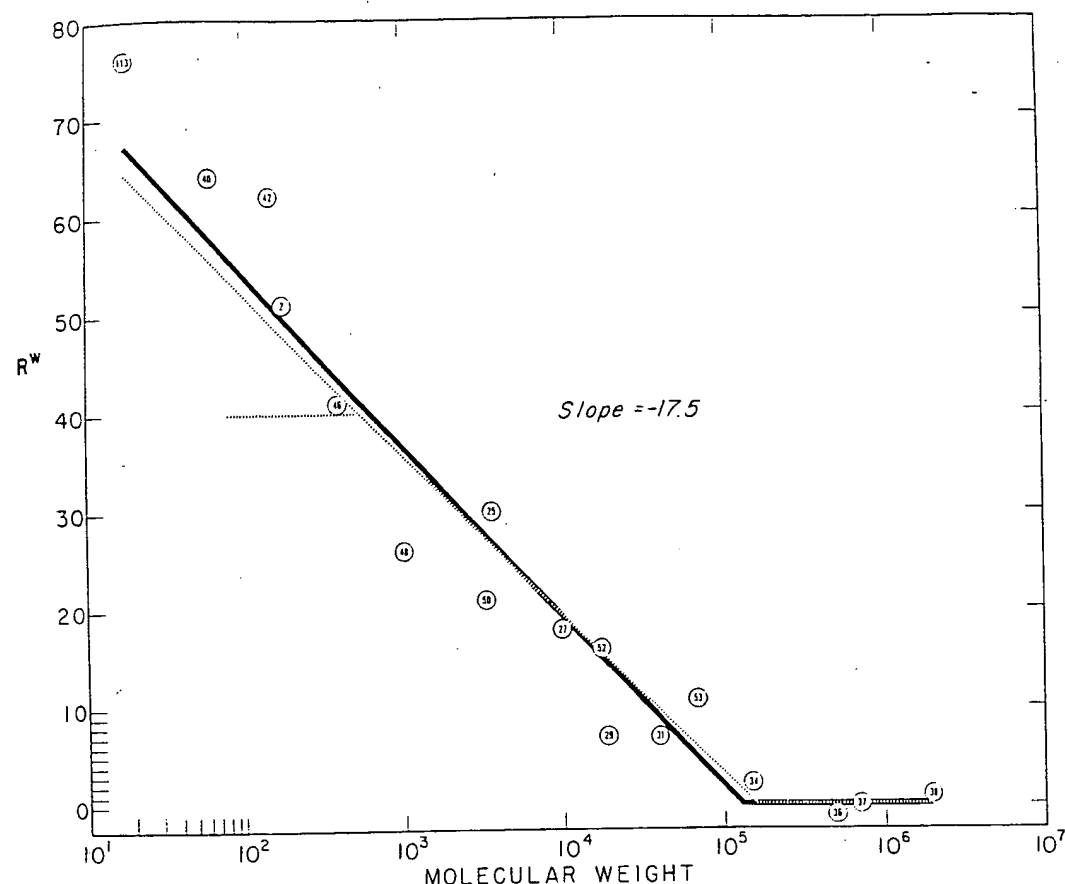


FIG. 2. Correlation between the molecular weight of dextrans and glycols and the uptake by germinated spores. The numbered points correspond to the compounds listed in Table 4. The lines were plotted by the method of least squares, with the points weighted according to the number of determinations. Points for glucose (no. 2) and water (no. 113) are plotted for reference; if these points are included, the slope becomes -19.2. The broken line represents the corresponding regression line for dormant spores (Fig. 2 in Gerhardt and Black, 1961).

be produced on the photographic emulsion as an artifact of overexposure or from the dye coupling onto the spore periphery. Since both photographs (Fig. 3A and 3C) were exposed for the same time, it appeared that a halation effect was produced by dye conjugating with spore coat polypeptide. Upon germination, the polypeptide was exuded (Powell and Strange, 1953) and a change in permeability allowed the dye to penetrate to the core, there to couple with intracellular protein. Fluorescence thus originated from a dormant spore over a comparatively large area, whereas the origination of light from a germinated spore, which lacks peripheral peptide, approximated a point source, as observed.

Uptake by chemically and physically treated

spores. Treatments that are believed to disrupt membranes might also effect a rise in solute uptake by spores. Organic solvents, for example, often have been thought to enhance cellular permeability, presumably by dissolving a lipid component in the membrane. Killing agents are also generally assumed to cause increased penetration of exogenous substances. Such treatments might be expected to simulate some of the effects brought about by spore germination. Several experiments with treated spores are summarized in Table 5. Dormant spores exposed either to a 5% (w/v) solution of phenol for 150 min or to a saturated solution of *n*-butanol for 5 min were not appreciably altered in their uptake of glucose or in their viability. When the spores were killed

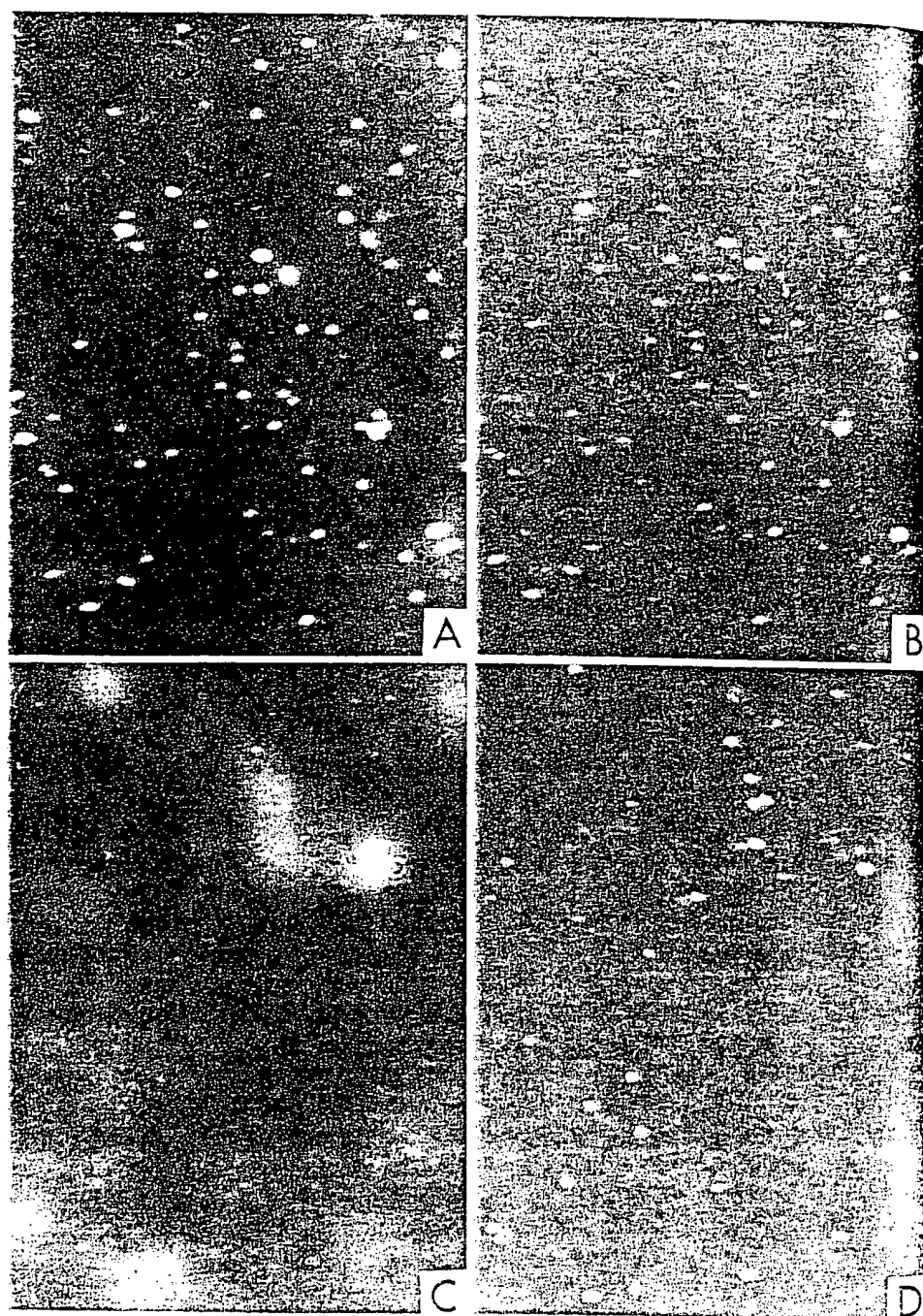


FIG. 3. Comparison of germinated and dormant spores suspended in a protein-fluorescent dye. A. Fluorescence micrograph of germinated spores. B. Reference darkfield micrograph of the same field as A. C. Fluorescence micrograph of dormant spores. D. Reference darkfield micrograph of the same field as C.

either by mild or extreme heat treatment, however, the uptake of glucose substantially increased and these values were comparable to the R value for glucose found for chemically germinated

cells (Table 3). Moreover, heat-killed spores fluoresced in the presence of TNS. I. Killing makes a cell completely permeable, as is usually presumed, then germination of a spore would

TABLE 5. Glucose uptake by chemically and physically treated spores

Conditions	Viability after the experiment	Uptake determination	
		S ^w Dextran	R ^w Glucose
Standard	%	%	%
Exposed 150 min to 5% (w/v) phenol solution	100	8	40
Autoclaved 60 min at 121 C	100	8	45
	0	7	52
Standard	100	9	34
Shaken 5 min in 8% (v/v) butanol solution	100	9	35
Heated 15 min at 100 C	0	9	51

seem from this limited comparison to cause much the same result.

DISCUSSION

Is the dormant spore selectively permeable to germination compounds? No. The experiments reported above indicate that, of the chemical germinants specific for *B. cereus* strain terminalis, glucose penetrated the spores no differently than other sugars, L-alanine was actually admitted to a lesser extent than most small molecules, and adenosine was taken up to a high but explainably nonspecific degree. Nor was the uptake altered when these substances were present in combination, at the temperature optimum for germination, or with spores which had first been mildly heated.

Does permeability change after the spore is germinated? Yes. A number of indirect signs and results of this change are commonly known and were listed above. Direct permeability measurements indicated that the germination of a spore results in some changes in permeability, notably to glucose and water, but the necessarily limited number of determinations permitted only a partial explanation in terms of the molecular variables previously found to govern solute uptake in dormant spores (Gerhardt and Black, 1961). Further evidence was provided by the penetration of a fluorescogenic dye into germinated spores. The occurrence of permeability changes attending germination should not, however, be taken as something unique to spores of bacteria. Pulvertaft and Haynes (1951) have

likened the breaking of bacterial spore dormancy to the hatching of amoebic cysts, and others (Fischer, 1897; Burke, 1923) have compared it to the moisture-induced germination of "impermeable seeds" in plants. Beyond qualitative analogy, however, are the detailed experiments of Sussman (1954), who has given quantitative evidence for a change in permeability of ascospores of *Neurospora tetrasperma* during germination.

The third question remains: does killing, which is generally presumed to make a cell completely permeable, simulate the permeability changes brought about by chemical germination? Yes. Dormant spores killed by heat were as permeable to glucose as chemically germinated spores.

The crux for understanding the observed changes in permeability may pertain to a greater water content in germinated than in dormant spores, since, to a considerable extent, the larger water-space value obtained for germinated spores accounts for the greater uptake of low mol wt solutes. A problem related to water content, moreover, is that of *water localization*. It may be that a central region is kept anhydrous in the dormant but not the germinated spore (Lewith, 1890; Rode and Foster, 1960). An effort to determine the exact content and location of the water in dormant and germinated spores now seems especially appropriate, not only for resolving the permeability problem but perhaps also for broaching the more general one of heat resistance.

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in a protein-fluorescogenic dye. A micrograph of the same field as A. micrograph of the same field as C.

3). Moreover, heat-killed spores in the presence of TNS. If killing is permeable, as is usually in germination of a spore would

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Sporulation and Germination 8

ROY H. DOI

1. INTRODUCTION

Since the 1950s bacterial sporulation in *Bacillus* has been recognized as a model system for analyzing differentiation in a prokaryotic organism. Earlier studies focused on the physiological and morphological changes which occurred during conversion of the vegetative cell into a heat-resistant, dormant spore. The physiological and morphological studies were complemented by the isolation of mutants which developed up to specific recognizable stages and were blocked in further development. However, the nature of spore-forming bacteria and the absence of suitable genetic and technical approaches precluded the precise analysis of the regulatory mechanisms which controlled sporulation. It was not possible to determine (1) which events regulated the initiation of sporulation, (2) what products were encoded by the sporulation and germination genes, (3) how genes were expressed in a temporally regulated fashion, (4) what the relationship was between catabolite repression and the initiation of sporulation, (5) what temporally regulated genes were essential for sporulation, (6) what transcriptional mechanism was required for the expression of sporulation genes, and (7) how the morphologically complex spore structure was assembled.

With the recent advent of recombinant DNA technology, the investigation of the regulatory events and the mechanisms of sporulation and germination in *Bacillus subtilis* has taken a dramatic upswing. Although the questions posed above have not been answered to date, the rapid progress being made currently will surely provide numerous answers and fulfill the expectations of those investigators who initiated the earlier studies on sporulation.

The earlier basic information concerning sporulation and germination is being complemented by information obtained by the new technology to provide a better understanding of the regulation of gene expression during growth and development.

Rapid progress has been made in the cloning and characterization of sporulation genes (*spo*), the isolation and characterization of forespores, the determination of the properties and specificities of RNA polymerase holoenzymes, the under-

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tanding of the factors which affect the initiation of sporulation, and the elucidation of the relationship between *spo0* genes, RNA polymerase, and catabolite repression. This chapter reviews and discusses some of these recent advances along with related information that can be utilized for further basic and applied research. A particular emphasis will be placed on evaluating our current understanding of the regulatory mechanisms which control the initiation of sporulation.

Several reviews which are pertinent to this discussion include the compilation of the latest genetic linkage map of *B. subtilis* (Piggot and Hoch, 1985; Zeigler and Jean, 1985); a summary of sporulation and germination genes (Piggot and Hoch, 1985); a review of the genetics of germination (Moir *et al.*, 1985); a description of several new methods to study developmental genes (Youngman *et al.*, 1985); a discussion of temporal gene expression in *B. subtilis* (Doi, 1984); and analysis of the initial events of bacterial sporulation (Freese, 1981); a description of the biochemistry of sporulation and germination (Setlow, 1981); and a discussion of the genetics of sporulation and germination (Piggot and Coote, 1976; Losick *et al.*, 1986).

1. PHYSIOLOGICAL AND MORPHOLOGICAL CHANGES ACCOMPANYING SPORULATION AND GERMINATION

A brief description of the physiological and cytological changes which occur during sporulation is presented as a background to the discussion of the various regulatory mechanisms currently being analyzed.

1.1. Response to Nutrient Deprivation

Nutrient deprivation of *Bacillus* triggers several cellular responses that attempt either to prevent starvation or to prepare the cell for a long period when nutrients are not available. The lack of readily metabolizable carbon, nitrogen, and phosphorus compounds are known to induce these events (Schaeffer *et al.*, 1965b). One early response of the cell to lowered concentrations of nutrients is to synthesize flagella which convert essentially nonmotile, linked, logarithmic phase cells into highly motile, individual, early stationary phase cells. This increased motility allows the cell to search for nutrients and respond chemotactically to a source of nutrients (Ordal *et al.*, 1985).

In addition to increased motility, the absence of nutrients derepresses a number of genes which code for intracellular and extracellular enzymes. The intracellular enzymes include enzymes of the tricarboxylic acid (TCA) cycle (Hanson *et al.*, 1963) and other carbon utilization enzymes (Nihashi and Fujita, 1984). When *Bacillus* is grown on glucose, organic acids such as pyruvate and acetate accumulate in the medium and reduce the pH of the medium to about 5–6 (Nakata and Talvorson, 1960). After glucose is depleted and the TCA enzymes are derepressed, the organic acids are utilized as a source of carbon and the pH of the medium rises to about 7. Thus a readjustment of the intracellular metabolic enzymes allows the cell to utilize other sources of carbon.

Besides synthesizing a new array of intracellular enzymes, the genes for extracellular enzymes are derepressed during nutrient starvation. These secreted en-

zymes include proteases, nucleases, amylases, phosphatases, and other hydrolytic enzymes (Schaeffer, 1969; see Chapter 11). These extracellular enzymes search out and attack suitable biopolymers and substrates in the microenvironment and furnish the cell with suitable monomeric nutrients.

If the cell is successful in swimming to another source of nutrients or its extracellular enzymes are able to hydrolyze sufficient amounts of nutritious biopolymers in its microenvironment, then the cell continues to grow. However, if these initial responses fail to provide the cell with required nutrients, the cell then initiates another sequence of events which results in the formation of a dormant spore. Spores are capable of remaining dormant for many years under the appropriate conditions; however, these same spores can germinate within minutes when suspended in a suitable nutrient-rich environment.

Although sporulation is a dramatic and morphologically identifiable stage in the life cycle of *Bacillus*, it could be considered as a "last resort" response of the cell to the very stressful situation of nutritional deprivation. Many bacteria also respond to starvation by growing flagella and derepressing genes for catabolic functions, but do not sporulate. Many of these bacteria live in a less hostile environment than species of *Bacillus* which tend to live in nutritionally poor environments such as soil and dry grass. The special ability of *Bacillus* species to sporulate allows these microorganisms to tolerate exposure to poor nutritional conditions for prolonged periods of time.

2.2. Sporulation Stages

Sporulation has been divided into seven stages based on the cytological changes which occur during spore formation (Schaeffer *et al.*, 1965a; Ryter *et al.*, 1966) (Fig. 1). Stage 0, which is a preparatory stage for sporulation, occurs at the end of the logarithmic phase of growth or at T_0 (T_1 , T_2 , T_3 , etc., represent the time in hours after the end of the log phase). The sporulation process usually takes about 6–8 hr under laboratory conditions of growth and thus the stage of development and the time in hours is rather similar, e.g., stage IV occurs at about T_4 .

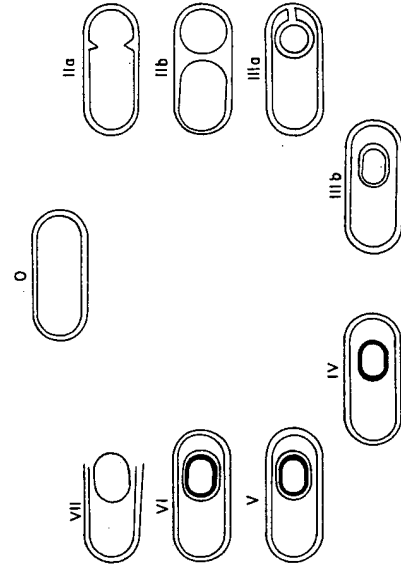


Figure 1. Schematic illustration of sporulation stages in *B. subtilis*. Proceed clockwise from stage 0, which occurs at the end of the logarithmic phase of growth. See text for description of stages.

phase are of particular interest, since their appearance indicates that either catabolite-repressed or specific developmental genes have been expressed. The expression of catabolite-repressed genes allows the cell to reorient its metabolism, e.g., by activating the tricarboxylic acid cycle (Hanson *et al.*, 1964) or by secretion of extracellular enzymes (Schaeffer, 1969). Release from catabolite repression is therefore one of the earliest responses to nutrient deprivation. One of the shortcomings of looking at overall protein patterns is that they can demonstrate only the relative extent of changes which are occurring. Specific protein changes cannot be identified unless the protein spots have been identified previously, as is the case in *E. coli* (Neidhart *et al.*, 1983). Unfortunately, this is still not the case for *B. subtilis* proteins.

2.4. Separation of Forespore and Mother Cell

A more specific way to study the protein patterns of sporulating cells is to examine the proteins present in the mother cell and in the forespore. This should provide information on the location of particular proteins during the differentiation process. For this purpose a method is required for isolating intact forespores from the mother cell after the engulfment stage so that comparisons may be made between their protein patterns. However, one of the difficulties with analyzing the proteins of isolated forespores is that it cannot be said for certain whether the genes for these proteins were expressed in the forespore or in the mother cell prior to forespore formation (stage 0). In the latter case the proteins would be entrapped in the forespore during forespore formation and subsequent engulfment. Also, it is possible that a protein synthesized in the mother cell could be transported across the double membrane into the forespore. Although these limitations exist, several investigators have obtained data which suggest that both the mother cell and the forespore chromosomes are actively and differentially expressed during sporulation.

3. SPORULATION-RELATED PROTEINS OF THE FORESPORE AND MOTHER CELL

The specific types of proteins which have been investigated as they relate to the sporulation process include various intracellular enzymes, membrane proteins, spore coat proteins, small acid-soluble spore proteins, and extracellular enzymes.

3.1. Forespore and Mother Cell Proteins

The initial morphological evidence for the onset of sporulation is associated with the asymmetrical synthesis of a forespore membrane during stage II. This process leads ultimately to the compartmentalization of the cell into a small forespore nestled within the mother cell (stage III or engulfment). Cellular events prior to stage II control this important step, since *spo0* mutants (blocked at stage 0) do not form a forespore membrane (Ryter *et al.*, 1966).

There has been a question for some time as to whether the forespore is differentially expressing genes for specific sporulation functions during its maturation

process (Warren, 1968; DeLencastre and Piggot, 1979). This question has been partially answered by the development of suitable methods for the isolation of intact forespores at various stages of sporulation (Andreoli *et al.*, 1973; Ellar and Postgate, 1974). The availability of intact forespores has allowed a comparison of the enzymes and proteins synthesized within the mother cell and forespore cytoplasm.

The isolated forespore is able to synthesize proteins *de novo* as demonstrated with forespore extracts and intact forespores (Andreoli *et al.*, 1975; Watabe *et al.*, 1981); moreover, more RNA synthesis occurs per unit of cell weight in the forespore than in the mother cell (Nakayama *et al.*, 1980). Although there is a high rate of protein turnover in the mother cell (Kornberg *et al.*, 1968), no protein turnover was detected in the forespore (Eaton and Ellar, 1974).

The data obtained by two-dimensional isofocusing-sodium dodecyl sulfate polyacrylamide gel electrophoresis (O'Farrell, 1975) indicate that the pattern of proteins in the early forespores resembles that found in the mother cell, but as the forespore matures, the patterns found in the two cytoplasmic diverge. Some genes are expressed in both compartments, while others are expressed either in the mother cell or in the forespore. These results are summarized in Table I.

Some spore components such as alanine dehydrogenase are synthesized in the mother cell compartment and then are transported to the developing spore—perhaps to its outer surface layers (Andreoli *et al.*, 1973). The enzymes which synthesize dipicolinic acid are found only in the mother cell, but dipicolinic acid is

Table I. Site of Protein Synthesis in the Mother Cell and Forespores^a

Protein	Mother cell	Forespore	References
Alanine dehydrogenase	+	—	Andreoli <i>et al.</i> , 1973
Dipicolinic acid synthetase	+	—	Andreoli <i>et al.</i> , 1975
Dihydrodipicolinic acid synthase	+	—	Andreoli <i>et al.</i> , 1975
Quinolinate phosphoribosyltransferase	+	—	Andreoli <i>et al.</i> , 1975
Spore coat precursor	+	—	Munoz <i>et al.</i> , 1978; Aronson and Pandey, 1978; Jenkinson <i>et al.</i> , 1981
Alanine aminotransferase	+	+	Andreoli <i>et al.</i> , 1973
Aspartate aminotransferase	+	+	Andreoli <i>et al.</i> , 1973
NADH oxidase	+	+	Andreoli <i>et al.</i> , 1973
NADH-DCIP ^b oxidoreductase	+	+	Andreoli <i>et al.</i> , 1973
Small acid-soluble spore proteins (SASPs)	—	+	Singh <i>et al.</i> , 1977; Tipper <i>et al.</i> , 1981
SASP protease	—	+	Singh <i>et al.</i> , 1977
Glucose dehydrogenase	—	+	Fujita <i>et al.</i> , 1977; Nakayama <i>et al.</i> , 1980; Chaudry <i>et al.</i> , 1984

^a + means that synthesis occurs and — means synthesis does not occur in the cell compartment. Many, but not all, of these data are based on the analysis of isolated forespores.
^b DCIP = 2,6 dichlorophenol indophenol.

found only in the dormant spore (Andreoli *et al.*, 1975). Thus small metabolites appear to be able to cross the double membrane of the developing forespore.

Since spore coat precursor proteins are made prior to engulfment (stage III), it is likely that spore coat proteins are synthesized only in the mother cell compartment and processed either during or after they have been transported to the outer surface of the maturing spore (Munoz *et al.*, 1978; Aronson and Pandey, 1978; Pandey and Aronson, 1979). Other proteins, such as glucose dehydrogenase (Fujita *et al.*, 1977; Nakayama *et al.*, 1980) and small acid-soluble spore proteins (SASPs) (Singh *et al.*, 1977; Tipper *et al.*, 1981), are synthesized only within the forespore. The RNA polymerase σ factor (Haldenwang *et al.*, 1981; see Section 6.1) is synthesized as early as T₂ in the mother cell (Fukuda and Doi, 1977) but is found primarily within the forespore cytoplasm (Nakayama *et al.*, 1980). Since this protein is synthesized in a precursor form (Trempey *et al.*, 1985a; LaBell *et al.*, 1987), it is possible that the precursor form facilitates its transport into the forespore from the mother cell cytoplasm. Thus these examples indicate strongly that differential gene expression occurs within both the mother cell and forespore compartments during sporulation and that some type of developmental mechanism regulates the final physical disposition of the protein products. The elucidation of this latter mechanism will test the imagination and skill of future experimenters.

Regulation of Activity of Forespore Enzymes

Mechanisms exist in the forespore which regulate the activity of its enzymes. One example which has been noted is the synthesis and accumulation of SASPs, which are synthesized during stages IV–V at the same time as the specific SASP protease (Singh *et al.*, 1977). The SASP protease efficiently degrades SASPs during germination (Setlow, 1975a), but is obviously not active during sporulation when large amounts of SASPs are synthesized and stored in the developing spore.

Another case involves the synthesis and storage of high levels (up to 5% of spore dry weight) of 3-phosphoglyceric acid (3-PGA) in the developing spore. In some *Bacillus* species 3-PGA is one of the main energy reserves of the dormant spore (Setlow, 1981). During germination 3-PGA is utilized in the first 10–15 min to yield energy and acetate for the germinating cell. This compound is accumulated during sporulation in the presence of significant levels of the enzymes which can catabolize it. Thus, as with the SASPs, 3-PGA must be inaccessible to phosphoglycerate mutase and the other degradative enzymes (Singh and Setlow, 1979a,b). Therefore mechanisms exist in the developing forespore which either inhibit the activity of the various catabolic enzymes or separate the various enzymes from their substrates by some type of compartmentalization until the germination mechanism is activated.

3.2. Forespore Septum Proteins

Since the synthesis of the forespore septum plays a critical role in the initial stages of sporulation, it is highly likely that it has some unique features which distinguish it from the mother cell cytoplasmic membrane.

By use of two-dimensional electrophoresis (O'Farrell, 1975), Andreoli *et al.*

(1981) demonstrated the presence of at least 260 membrane proteins in late exponential and stage III (engulfment) cells. Approximately 50 proteins present in stage III cell membranes were absent from the membranes of late exponential phase cells. In comparing specific membrane proteins they observed a series of protein increases, decreases, deletions, and the appearance of totally new proteins. Many of the new proteins were already present in the forespore septum (stage II) and very few new proteins were added to the membrane during the engulfment process (stage III).

Subsequently, Chui *et al.* (1984) and Andreoli (personal communication) found six new major membrane proteins that appeared during asymmetrical septum assembly and two new major proteins were observed only during engulfment membrane formation. The membrane of a *spoIIA* mutant, which forms an asymmetrical septum but fails to assemble engulfment membranes, contained only the six membrane proteins found in the asymmetrical septum and lacked the two engulfment membrane proteins. The occurrence of the eight new major membrane proteins was independent of the conditions used for obtaining sporulation. Thus these observations indicate that specific proteins may be required for the formation of the asymmetrical septum and for engulfment of the forespore.

In this regard, studies on intergenic suppression of *spoI* mutants (Sharrock *et al.*, 1984; Leung *et al.*, 1985) and the partial homology of the *spoOA* protein product to the *ompR* gene product (Ferrari *et al.*, 1985b) indicate that membrane functions may play key roles during forespore septum formation and the initiation of sporulation. Wayne *et al.* (1981) and Wayne and Leighton (1981) showed that mutationally induced *Spots* phenotypes and the cerulinin-induced *Spots* phenotype (cerulinin is an inhibitor of fatty acid synthesis) can be relieved by the presence of precursors of membrane biosynthesis. Also, a suppressor mutation *rev4* can restore sporulation in *Spots* mutants, in *Spots* phenotypes induced in wild-type *B. subtilis* by ethanol and phenethyl alcohol, and in cerulinin-treated wild-type cells (Sharrock and Leighton, 1982). Therefore the *rev4* suppressible sporulation phenotypes appear to be associated with defective membrane structure or function.

Another intergenic suppressor of *spoI* phenotypes called *rutA* (Sharrock *et al.*, 1984) can prevent the disruption of sporulation by aliphatic alcohols in a manner similar to that of *rev4*. Since *rutA* is now considered to be a mutation in the *spoOA* gene (Ferrari *et al.*, 1985b), it appears that *spoI* genes are involved in controlling membrane functions.

The further characterization of the sporulation-related membrane proteins and their genes should shed light on their role in the assembly and functions of forespore septum and engulfment membrane. Furthermore these studies should clarify the role, if any, of *spoOA* on the regulation of membrane protein genes.

3.3. Spore Coat Proteins

One of the ironies of studying sporulating cells has been the difficulty in identifying sporulation-specific proteins. Many activities associated with sporulation may turn out to play no direct role in sporulation itself. However, the spore coat proteins are obviously good candidates for study as sporulation-specific gene products, since their genes are expressed only during sporulation and their products are

clearly spore components. The coat proteins may comprise from 40 to 80% of the total spore protein (Spudich and Kornberg, 1968; Munoz *et al.*, 1978; Goldman and Tipper, 1978; Aronson, 1981) and therefore are major components of the spore and major products of the protein-synthesizing machinery of the sporulating cell.

There has been, however, some difficulty in isolating and characterizing spore coat proteins since they tend to be insoluble and require rather harsh extraction treatments (Pandey, 1980). The total extraction of spore coat proteins requires a combination of detergents, protein-denaturing agents, high pH conditions, and agents which disrupt disulfide bonds. Since these proteins are nonenzymatic, they are usually assayed immunologically, which may or may not be specific depending on the antigen preparation used for antibody production. In spite of these inherent difficulties, the spore coat proteins have been well characterized in recent years.

The spore coat is a complex structure consisting of at least 12 different polypeptides (Goldman and Tipper, 1978; Pandey and Aronson, 1979; Pandey, 1980; Jenkinson *et al.*, 1981) and is composed of several layers of proteins which are laid down successively during spore maturation (Jenkinson *et al.*, 1981; Jenkinson, 1981; Jenkinson and Sawyer, 1981).

At least some of the spore coat proteins are made as early as stage II, i.e., before forespore engulfment (Wood, 1972; Munoz *et al.*, 1978; Pandey and Aronson, 1979). They are made as precursor coat proteins (Munoz *et al.*, 1978; Pandey and Aronson, 1979), which are processed to the mature form presumably by specific intracellular proteases (Kerjan *et al.*, 1979; Srivastava and Aronson, 1981; Jenkinson and Lord, 1983) either before or after they are assembled on the surface of the developing spore. This makes the analysis of late sporulation processes somewhat more difficult, since some of the events occurring after stage IV require the processing and assembly of proteins formed earlier in stages II and III (Dion and Mandelstam, 1980; Jenkinson *et al.*, 1980). Also the later stage IV–VI phenotypes may be the result of a mutation which affects the synthesis of a protein as early as stage II. Thus some caution must be taken in interpreting the phenotypes of so-called late stage mutants.

Several spore coat mutants have been found and designated as *gerE* (Moir *et al.*, 1979), *spoVIA* (Jenkinson, 1981), and *spoVIB* (Jenkinson, 1983). These mutants have abnormal spore coats, and may have altered heat resistance and germination properties. The *spoVIA* mutant spore lacks a 36,000-Da polypeptide normally found in the outermost layer of the mature spore, has a delayed response to germinants, and is sensitive to lysozyme. It is, however, fully heat-resistant (Jenkinson, 1981). The *spoVIB* mutant has a 12,000-Da protein which is assembled abnormally into the spore coat; this mutant sporulates and germinates more slowly than normal cells and is therefore a spore coat assembly mutant (Jenkinson, 1983). The *gerE* mutant produces spores that are resistant to heat- and organic solvents, are lysozyme-sensitive, and have an incomplete coat structure (Moir, 1981). The coat lacks a number of polypeptides but has four polypeptides not present in wild-type spore coats (Jenkinson and Lord, 1983). The *gerE* mutant also fails to synthesize an intracellular protease B at stage V which is normally incorporated into spore outer layers. The highly pleiotropic nature of this mutation suggests that the *gerE* locus codes for a regulatory product required for expression of late sporulation genes (Moir, 1981).

The analysis of spore coat proteins presents several intriguing questions:

(1) How do the spore coat proteins made early in sporulation survive the rigorous turnover of proteins which occurs in the mother cell during sporulation? Are the precursor forms resistant to intracellular proteases? Do the precursors become susceptible to protease cleavage only after assembly on the surface of the maturing spore? Is there one or several proteases that can process the precursors?

(2) How are the spore coat proteins transported to the developing spore surface and when are the precursor coat proteins processed to the mature form? Since the mature forms are rather insoluble at neutral pH and in aqueous solutions, it appears likely that soluble precursors are assembled on the developing spore surface prior to final processing.

(3) What factors regulate the expression of the spore coat protein genes? Since some of these proteins are made very early in sporulation (stage II) and some are made later (stage V–VI) (Jenkinson and Sawyer, 1981), it is possible that different forms of the transcriptional machinery may transcribe these coat protein genes.

(4) What factors determine the order of assembly of the many layered spore coat complex? Is the time of synthesis of each of the spore coat proteins sufficient to determine the sequential layering of the spore coat proteins? Are there assembly proteins which facilitate the proper layering process? Is there sufficient information in the coat protein molecule itself to control the assembly process, i.e., does self-assembly occur?

Some of these questions will be answered soon, since spore coat protein genes have been cloned (Donovan *et al.*, 1987) and can be used as probes for measuring spore coat gene expression. The elucidation of the mechanisms for processing and the sequential assembly of the spore coat proteins on the surface of developing spores will require a substantial amount of effort. It would be quite a feat if an *in vitro* system could be developed to provide all the coat protein precursors, processing enzymes, and a forespore for the assembly of the spore coat complex perhaps in a manner analogous to that of the less complex phage particle.

3.4. Small Acid-Soluble Spore Proteins

The small acid-soluble spore proteins (SASPs), whose molecular masses range from 12,000 to 15,000, have been studied extensively by Setlow and his colleagues (Fliss *et al.*, 1985). These proteins, which comprise 10–20% of the dormant spore protein, are degraded during germination and provide amino acids for the germinating spore (Setlow *et al.*, 1980; Setlow, 1981; Fliss *et al.*, 1985). The spore protease which initially cleaves these proteins during germination has a very high sequence specificity and attacks a target site with the following amino acid sequence (Dignam and Setlow, 1980; Setlow *et al.*, 1980; Yuan *et al.*, 1981; Fliss and Setlow, 1984b):



The cleavage occurs between the Glu and (Phe or Ile) residues.

The SASPs are synthesized in the forespore throughout the sporulation period (Dignam and Setlow, 1980) and their expression is under transcriptional control (Setlow, 1981). Several SASPs have been completely sequenced (Setlow and Ozols, 1979, 1980a,b; Fliss and Setlow, 1985; Fliss *et al.*, 1986; Connors *et al.*, 1986). Seven

SASP genes have been cloned from *B. megaterium* (Curiel-Quesada *et al.*, 1983; Curiel-Quesada and Setlow, 1984; Fliss and Setlow, 1984a,b; Fliss *et al.*, 1985; Hackett *et al.*, 1986) and from *B. subtilis* (Hackett and Setlow, 1987); it is likely that most *Bacillus* species will contain multiple SASPs (Yuan *et al.*, 1981; Johnson and Tipper, 1981; Fliss and Setlow, 1985; Sun and Setlow, 1987). By using a cloned SASP C-3 from *B. megaterium* as a hybridization probe, Fliss and Setlow (1985) were able to demonstrate that the C-3 gene mRNA was made prior to the formation of dipicolinic acid and that at least four SASP genes were expressed in parallel during sporulation.

Several other interesting features of the *B. megaterium* SASP genes have emerged:

- (1) The SASP genes are the first extended, divergent, multigene family which has been described in prokaryotes (Fliss *et al.*, 1985).
- (2) The upstream regions of the SASP genes have promoters which are similar in sequence in their -35 and -10 regions and may be transcribed by the same RNA polymerase holoenzyme (Fliss *et al.*, 1985). The SASP genes are expressed, however, at different levels.
- (3) The ribosome binding sites of the SASP mRNAs are stronger than those usually found in *E. coli* mRNAs (Fliss *et al.*, 1985).
- (4) The genes are monocistronic since they all have transcription termination sites just downstream of the translation stop codon. The genes for this multigene family are scattered throughout the chromosome (Fliss *et al.*, 1985).
- (5) The DNA sequences of the SASP genes and the amino acid sequence of the SASPs are highly conserved, with about 50% of the amino acid residues conserved and the greatest amino acid changes occurring in the N- and C-terminal regions of the proteins (Fliss *et al.*, 1985). The *B. megaterium* SASPs are related immunologically both among *B. megaterium* SASPs and with SASPs from *B. subtilis* (Connors and Setlow, 1985) and *B. cereus* (Fliss *et al.*, 1985). Thus the genes for these proteins appear to be very highly conserved among different species of *Bacillus*.
- (6) The SASPs are in close proximity *in vivo* to the DNA of spores, since they can be crosslinked to DNA by ultraviolet irradiation of whole spores but not of spore extracts (Setlow and Setlow, 1979). It has been proposed that the SASPs may be involved in the resistance of spore DNA to UV light (Setlow, 1975b); since they can bind to DNA *in vitro* (Setlow, 1975a,b), they accumulate in developing spores at the time they become resistant to UV light, and their absence makes spores more sensitive to UV irradiation (Mason and Setlow, 1986, 1987).

Although the functions of the SASPs, beyond that of being storage proteins and possible DNA-binding proteins in the dormant spore, remain obscure, the analysis of the SASP gene family is likely to provide new basic information about the regulation of expression of developmental genes. Since they are expressed in parallel during mid-sporulation, SASP genes provide a model system for studying the regulation of coordinate transcription of widely scattered genes. The conserved regions of the promoters for the SASP genes are not homologous to the previously published consensus promoter sequences (Moran *et al.*, 1982; Doi, 1982a) and may represent promoters recognized by a heretofore unrecognized form of RNA polymerase holoenzyme.

The fact that SASP genes are expressed only in the forespore (Singh *et al.*, 1977) also indicates that differential transcription is occurring in the forespore and

that this is a clear case of compartmentalized transcription and translation in a prokaryotic cell. The question therefore arises as to whether SASP genes have promoters which are recognized by a RNA polymerase occurring only in the forespore? Further investigation of this system should reveal the metabolic signals which control transcription in the forespore, the factors required for specific transcription of genes expressed only in the forespore, the mechanism which determines the relative expression of the SASP genes, and the extent of total gene expression in the forespore relative to that of the mother cell.

A further comparison of the base and amino acid sequence of the SASP genes and their protein products may reveal why some regions of the genes are highly conserved, how these genes have become scattered throughout the genome, what the evolutionary pattern of this gene family is in the various *Bacillus* species, why the genes are monocistronic, and what the functions of SASPs are. Thus for a multiplicity of reasons the SASP family gene system has high potential for providing greater understanding of the sporulation process and the properties of spores.

3.5. Extracellular Enzymes

Another class of proteins which are synthesized after growth has ceased includes the extracellular enzymes (Table II). Gel electrophoresis patterns of extracellular proteins present at T₅ show more than 30 bands (Porter and Mandelstam, 1982). These enzymes are synthesized at the initial stages of sporulation and secreted into the growth medium. Most of the genes for these proteins as well as sporulation genes appear to be repressed by mechanisms similar to catabolite repression, since they are not expressed in the presence of excess glucose or nitrogen sources. The genes for these extracellular proteins are of particular interest for biotechnology (see Chapter 11).

The signal peptides coded by these *Bacillus* extracellular enzyme genes (Kroyer and Chang, 1981; Neugebauer *et al.*, 1981; Palva *et al.*, 1982; Ohmura *et al.*, 1983; Wells *et al.*, 1983; Wong *et al.*, 1984) are similar in composition to signal peptides reported previously in other prokaryotes and in eukaryotes (Kreil, 1981) and have the classic short, charged, N-terminal peptide followed by a long sequence of hydrophobic amino acids and a signal peptidase cleavage sequence (Perlman and Halvorson, 1983; see Chapter 5). The *Bacillus* signal peptides appear on average to be slightly longer than those found in *E. coli*. A number of investigators are attempting to use *B. subtilis* promoters and signal peptides to secrete foreign gene products from *B. subtilis* (see Doi, 1984, for review).

One long-standing question has been whether all enzymatic functions that were derepressed during sporulation had a direct relationship to spore formation. There now appears to be an overall regulatory function which controls the expression of genes for extracellular enzymes and for sporulation. This is best illustrated in the case of the *B. subtilis* extracellular alkaline serine protease (subtilisin) gene (*aprA*). The expression of this gene and the early sporulation genes is controlled by *spo0A* (Hoch and Spizizen, 1969). A mutation in this locus prevents the expression of the subtilisin gene and also blocks sporulation at stage 0. This pleiotropic effect was initially interpreted as an indication that the subtilisin gene was directly associated with sporulation. However, deletion mutations of the subtilisin gene have been obtained recently and these mutant cells are still capable of sporulating as efficiently

Table II. Extracellular Enzymes of *Bacillus* Species

Enzyme and <i>Bacillus</i> species	References ^a
Subtilisin (serine protease) <i>B. subtilis</i>	Wong <i>et al.</i> (1984) Stahl and Ferrari (1984)
<i>B. amyloliquefaciens</i>	Wells <i>et al.</i> (1983) Vasantha <i>et al.</i> (1984a)
Neutral protease <i>B. subtilis</i>	Yang <i>et al.</i> (1984)
<i>B. amyloliquefaciens</i>	Vasantha <i>et al.</i> (1984b)
<i>B. stearothersmophilus</i>	Shimada <i>et al.</i> (1985) Fuji <i>et al.</i> (1983)
Esterase <i>B. subtilis</i>	Mamas and Millet (1975) Karmazyn-Campelli and Millet (1981)
α -Amylase <i>B. subtilis</i>	Yang <i>et al.</i> (1983) Yamazaki <i>et al.</i> (1983) Shinomiya <i>et al.</i> (1984) Palva (1982)
<i>B. amyloliquefaciens</i>	Takinen <i>et al.</i> (1983)
<i>B. licheniformis</i>	Stephens <i>et al.</i> (1984b) Ortlepp <i>et al.</i> (1984)
<i>B. stearothersmophilus</i>	Tsukagoshi <i>et al.</i> (1984)
Ribonuclease <i>B. subtilis</i>	Nishimura and Nomura (1958) Coleman and Elliott (1965) Nakai <i>et al.</i> (1965)
Phosphodiesterase <i>Bacillus</i> sp. <i>B. subtilis</i>	Jacobsen and Rodwell (1972) Nakai <i>et al.</i> (1965)
Deoxyribonuclease <i>B. subtilis</i>	Nakai <i>et al.</i> (1965) Akriegg and Mandelstam (1978)
Alkaline phosphatase <i>B. licheniformis</i>	Hulett (1984) Hulett <i>et al.</i> (1985) Glenn and Mandelstam (1971)
<i>B. subtilis</i>	Fouet <i>et al.</i> (1982)
Sucrase <i>B. subtilis</i>	Sloma and Gross (1983)
β -Lactamase <i>B. cereus</i>	Mezes <i>et al.</i> (1983)
Penicillinase <i>B. licheniformis</i>	Kroyer and Chang (1981) Neugebauer <i>et al.</i> (1981)

^a These are primarily recent papers concerned with cloning of the genes.

as wild-type cells (Kawamura and Doi, 1984; Yang *et al.*, 1984; Stahl and Ferrari, 1984). Thus although the expression of the subtilisin gene and sporulation genes are under a common control mechanism (*spo0A*), subtilisin is not an essential part of the sporulation process.

These results support the idea that the *spo0A* mutation affects an overall regulatory function which controls the expression of many genes including those for extracellular enzymes and for sporulation, and that these functions are in different pathways and not in a single linear pathway leading to sporulation.

4. METHODS FOR THE ANALYSIS OF SPORULATION AND GERMINATION GENES

With the new recombinant DNA technology (see Chapter 6), several approaches have been developed to identify sporulation and germination loci. This should allow the identification and characterization of the approximately 42 sporulation loci or operons, which were calculated to be present on the *B. subtilis* chromosome by Hranueli *et al.* (1974), and many other germination and outgrowth loci. The complexity of the sporulation and germination process is illustrated by the identification of at least 60 genes and/or loci for sporulation (*spo*), germination (*ger*), and outgrowth (Piggot and Hoch, 1985). A multiplicity of other genes was also demonstrated which have an effect on the initial stages of sporulation (e.g., *rpoD*, *che*); several of these may function during both vegetative growth and sporulation. The exact nature and function of most of the *spo* and *ger* genes are still unknown. Nevertheless rapid progress is being made in cloning these genes and identifying their products, e.g., by homology with known proteins. Thus it would be very useful to have a full catalogue of sporulation and germination genes. Several methods have been developed recently which should facilitate the identification and characterization of these developmental genes.

4.1. Transposon-Directed Insertional Mutagenesis

Streptococcus faecalis transposon Tn917 has been used to create mutations in the *B. subtilis* chromosome; this has resulted in the isolation of various sporulation-defective phenotypes (Youngman *et al.*, 1984a,b; see Chapter 6, Sections 7 and 9).

An analysis of these transposon insertion mutants indicated that a variety of auxotrophs were obtained at a frequency of 5–8%. In addition, *spo* mutants were observed at a frequency of 0.1–0.5% (Youngman *et al.*, 1984b). Of the eight *spo* mutants identified, all eight insertional mutations were distinct from each other and two of the eight were identified as completely new *spo* sites which were not detected by previous selection methods. Thus this method has the potential for isolating many heretofore unidentified *spo* and *ger* loci (Youngman *et al.*, 1985).

Once a transposon insertional mutation has been located in a *spo* locus, it would be extremely useful to be able to isolate relatively quickly all or part of the *spo* locus for further analysis. Isolation of the transcription regulatory region of the locus would allow, for instance, an analysis of the promoter and other putative transcriptional regulatory sites of this developmental locus. The isolation of a portion of the

locus would also permit its use as a hybridization probe of a gene bank of *B. subtilis* and the subsequent identification of a clone which contained the entire locus.

Youngman *et al.* (1984a) developed a novel method for rapid cloning of portions of *B. subtilis* *spo* genes adjacent to Tn917 insertions in the chromosome. The overall rationale for their method is described in Chapter 6, Section 7.4.

This method has been used to isolate a portion of the *spoIIH* gene of *B. subtilis* and can potentially be used to isolate a portion of any gene in which Tn917 resides (Youngman *et al.*, 1984a-c; 1985).

4.2. Use of Gene Fusions for the Study of the Functions of Temporally Regulated and Sporulation Genes

Since the exact functions of most sporulation genes, even those that have been cloned, are still unknown, it is difficult to measure their activities *in vivo* or *in vitro*. Also, if the regulation of these genes is to be analyzed, a method is required to measure the expression of these genes in various genetic backgrounds and under different physiological conditions. If the gene product happens to be a structural protein without enzymatic activity, it becomes extremely difficult to measure directly the regulation of such a gene. One way to overcome this difficulty is to fuse the transcriptional regulatory regions of sporulation genes with a gene whose expression is readily detectable, e.g., an easily assayable enzyme. Then the expression of the sporulation gene can be monitored by assaying the activity of the enzyme. Two such general systems have been reported (Goldfarb *et al.*, 1981; Zuber and Losick, 1983; Youngman *et al.*, 1984b).

4.2.1. Gene Fusions with Tn9-Derived Chloramphenicol Acetyltransferase

In one case a fusion product was formed between the N terminus of a temporally regulated *B. subtilis* gene and the Tn9-derived chloramphenicol acetyltransferase (*cat*) gene present in plasmid pGR71 (Goldfarb *et al.*, 1981). pGR71 is a cointegrate shuttle plasmid which can replicate in either *B. subtilis* or *E. coli*. The gene fusion is made possible by having a promoterless Tn9-derived *cat* gene preceded by a *HindIII* insertion site present in the plasmid. When *HindIII* fragments were shotgun-cloned into this site, insertional activation of the *cat* gene occurred. Most of the products consisted of fusion polypeptides between N-terminal peptides of *B. subtilis* and the C terminus of the *cat* gene product. Since the *cat* gene has an *E. coli* ribosome binding site (RBS) which is not used efficiently by the *B. subtilis* translational machinery, all translation started at the *B. subtilis* RBS on the DNA insert (Goldfarb *et al.*, 1982).

The temporally regulated promoter isolated by this method (Goldfarb *et al.*, 1983a) was found to control the expression of the *B. subtilis* subtilisin gene (*aprA*) (Wong *et al.*, 1984). The translation of the fusion gene began at the ribosome binding site provided by the *aprA* gene and the in-phase translation continued through the leader region of the *cat* gene into the structural portion of the gene. The fusion products were enzymatically and immunologically active (Goldfarb *et al.*, 1982). The response of the gene to different temporal and physiological condi-

tions was measured by analyzing the CAT activity at different times of the growth curve and in the presence or absence of various nutritional factors (Goldfarb *et al.*, 1983a,b). The *cat* gene was expressed only during the stationary phase and with the same pattern as that observed for subtilisin synthesis. Although the *aprA* gene is temporally expressed at a similar time as the *spoII* genes and its expression is controlled by *spoOA* gene, deletion analyses have shown that the *aprA* gene is not required for sporulation (Kawamura and Doi, 1984). Other efficient promoters and fusion products (Wang and Doi, 1984) and the phenomenon of translational coupling have also been analyzed by this fusion system (Zaghloul *et al.*, 1985; Zaghloul and Doi, 1986, 1987). In principle, this system can be applied to the study of most sporulation-specific or sporulation-associated genes.

4.2.2. Fusion of *spoVG* with *E. coli lacZ*

In another type of construction, a system has been devised to study the regulation of the *spoVG* gene by fusing its transcriptional regulatory region to a gene whose product could be measured readily (Zuber and Losick, 1983). Furthermore the system was constructed to allow integration of the fused gene into the chromosome *via* an integrative plasmid (Haldenwang *et al.*, 1980; Ferrari *et al.*, 1983; Price *et al.*, 1983; see Chapter 6) and to study its expression *in vivo* under different regulatory conditions.

For this purpose the N terminus of *spoVG* was fused to the *E. coli lacZ* gene contained in the plasmid pZL207 (Zuber and Losick, 1983). This plasmid contained, in addition to the fusion gene, a *S. aureus* chloramphenicol resistance gene and a pBR322-derived replicon. This plasmid could be used as an integrative plasmid in *B. subtilis* by transforming into this organism and selecting for chloramphenicol resistance. Since no *B. subtilis* origin of replication (*ori*) was present, chloramphenicol resistance could only be expressed by integration of the plasmid into the chromosome by homologous recombination between the *spoVG* gene in the plasmid and in the chromosome. This integration provides a single copy of the regulatory region of *spoVG* fused to the *lacZ* gene.

The expression of the *spoVG* gene, as measured by β -galactosidase synthesis, was normal in a wild-type background. However, its expression was impaired in the presence of mutations in *spoOB*, *spoOC*, *spoOE*, *spoOF*, *spoOH*, *spoJ*, and *spoK* genes. Deletion experiments indicated that the regulation of *spoVG* by the *spoO* genes was exerted at or near the *spoVG* promoter region. Since *spoVG* has a σ^B promoter (see Section 6.1), these results also indicated that many *spoO* functions are required for the expression of genes controlled by σ^B promoters.

The normal expression of the *spoVG* gene was altered considerably if the fusion gene was present on a high-copy-number plasmid; the β -galactosidase activity was expressed considerably earlier in the growth phase and its induction was not impaired by *spoOB* and *spoOH* mutations. A high copy number of *spoVG* promoter also inhibited sporulation (Banner *et al.*, 1983). These results suggest that the amplified *spoVG* promoter may titrate a regulatory protein required early in sporulation and becomes limiting for the expression of *spoVG* and other sporulation genes (Zuber and Losick, 1983).

The use of fusion genes for structural protein genes such as spore coat pro-

teins, membrane proteins, and small acid-soluble spore proteins will allow a much more precise analysis of their expression and regulation during sporulation.

4.2.3. Transposon-Mediated Gene Fusions

As a further improvement of the fusion method, a transposon-mediated gene fusion system has been developed that allows the identification of temporally controlled and sporulation promoters and their genes (Youngman *et al.*, 1984a,b,c; see Chapter 6).

4.3. Cloning of Temporally Controlled Promoters

Although the isolation of a specific gene is most advantageous, there are occasions when the rapid isolation of a number of temporally controlled promoters may be desired. For this purpose a number of promoter expression probe plasmids have been developed for use in *B. subtilis*. These plasmids contain a replication origin (*ori*) for *B. subtilis*, a selectable gene without its promoter preceded by a suitable restriction site, and, often, an antibiotic resistance marker (see Chapter 6).

However, there is a slight complication when temporally regulated promoters are sought, e.g., sporulation or stationary phase promoters. Not all the expression probe plasmids work equally well in this situation. This is particularly true if an antibiotic gene is the selection gene. Since antibiotic resistance will not develop until the stationary phase with temporally regulated promoters, it is not possible to use antibiotic-containing plates for selection of the activated gene. If a selection gene is based on enzyme activity which provides a color and/or morphological signal, then it is possible to look for the late development of these signals relative to the early development of signals by promoters expressed during the exponential phase of growth. Kawamura *et al.* (1986) and Wang and Doi (1987) recently developed a subtilisin expression probe plasmid carried in a protease-deficient strain which develops halos on casein agar plates at about 10 hr with exponential phase promoters and at 20 hr for stationary phase promoters. Thus it is possible to distinguish developmental from exponential phase promoters quite readily with this expression probe plasmid. In addition, it has utility in the isolation of nutritionally controlled promoters, e.g., glucose-sensitive and insensitive promoters.

The use of these expression probe plasmids has led to the isolation of temporally regulated promoters (Goldfarb *et al.*, 1983a; Mongkolsuk *et al.*, 1983), catabolite-repressed and amino acid-controlled promoters (Goldfarb *et al.*, 1983b), as well as a large number of exponential phase promoters with differing degrees of promoter strengths and complexities (Goldfarb *et al.*, 1981).

4.4. Integrative Mapping and Cloning by Gene Conversion Techniques

Useful methods have been developed for mapping *B. subtilis* genes for which no mutations are known or for which assays are difficult, and for the rapid cloning of mutant genes when the wild-type gene has been cloned.

4.4.1. Integrative Mapping

For the mapping of a cloned *B. subtilis* gene in which no mutations are available for complementation studies, the rationale is to insert the gene into an *E. coli* plasmid containing an antibiotic resistance marker which can be expressed in *B. subtilis* and then transforming the plasmid into *B. subtilis*. Since no origin of replication (*ori*) exists for *B. subtilis*, the only antibiotic-resistant clones which will appear are those in which the plasmid has been integrated into the host chromosome by homologous recombination between the gene of interest in the plasmid and in the host chromosome. The antibiotic resistance marker is inserted close to the gene of interest in the *B. subtilis* chromosome and is then mapped by PBS1 transduction analysis using the set of mapping mutants developed by Dedonder *et al.* (1977) (see Chapter 4). This integrative mapping technique was first demonstrated for mapping of the 0.4-kilobase *spoVG* gene in *B. subtilis* by Haldenwang *et al.* (1980).

As another example of this type of analysis, the σ^{43} (σ^A) gene (*rpoD*) was cloned (Price *et al.*, 1983) by immunoscreening a *B. subtilis* gene bank constructed in λ gt WES (Ferrari *et al.*, 1981). Since no *rpoD* mutants were known at this time, integrative mapping was used to find the locus of the *rpoD* gene. The cloned *rpoD* was inserted into integrative plasmid pCP112 (Price *et al.*, 1983) which contained an *E. coli ori* from plasmid pBR327 (Soberon *et al.*, 1980) and the chloramphenicol resistance (*Cmr*) gene from PC194 (Horinouchi and Weisblum, 1982). This plasmid was transformed into *B. subtilis* and clones were selected for *Cmr*. By transduction analyses using *Cmr* as the selection marker, the location of *rpoD* was found to be at around 225° on the circular *B. subtilis* map (Price *et al.*, 1983; Price and Doi, 1985).

4.4.2. Gene Conversion

There are occasions when it is useful to clone several DNA fragments containing mutations at various locations in the same gene, e.g., to do sequence analyses of the DNAs to find the precise locations of the mutations. A rapid method has been developed for achieving this, based on gene conversion which is carried out actively by *B. subtilis*, presumably by its efficient mismatch repair mechanism (Chak *et al.*, 1982; Iglesias and Trautner, 1983; Kawamura and Doi, 1984).

This method depends initially on cloning a gene of interest on a *B. subtilis* plasmid such as pUB110. When a recombinant plasmid containing the wild-type gene is transformed into a host containing a chromosomal mutation of the gene, three types of clones will result. About 90–95% of the clones will represent the original transformant type in which the wild-type gene will be on the plasmid and the mutant gene will be in the chromosome; no gene conversion has occurred in this type. In the second type the clone will contain the wild-type gene in both the plasmid and in the chromosome; in this case the wild-type gene has replaced the mutant gene in the chromosome by gene conversion (we refer to this as “beaming down” of the wild-type gene from the plasmid to the chromosome). In the third type the clone will contain the mutant gene in both the plasmid and in the chromosome. In this case the mutant gene of the chromosome has replaced the wild-type gene in the plasmid by gene conversion (we refer to this as “beaming up” of the mutant gene from the chromosome to the plasmid).

The utility of the system is now evident, since if one selects for a mutant phenotype, one can find the mutant gene in the plasmid and the gene can then be isolated by plasmid screening for further analysis. However, the gene of interest has to be cloned first and be present on the plasmid before this technique is applicable. As an example of this type of analysis, the *crsA* mutations in the *rpoD* gene were beamed up from the chromosome of *B. subtilis* to plasmids and the exact locations of the *crsA* mutations within the *rpoD* gene were determined by base sequence analysis of the mutant genes residing in the plasmid (Kawamura *et al.*, 1985).

5. ISOLATION AND CHARACTERISTICS OF CLONED SPORULATION AND GERMINATION GENES

Over 50 genes and loci for sporulation and germination have been identified (Piggot and Hoch, 1985; Errington *et al.*, 1985). The characterization of these genes is essential for elucidating the regulatory and structural components of the sporulating cells. For this purpose a significant number of sporulation and germination genes have been cloned. Most of the cloning procedures have been based on finding DNA fragments which complement the *Spo⁻* phenotype. The cloned fragments have usually been carried on plasmids or temperate phage of *B. subtilis* (Iijima *et al.*, 1980; Kawamura *et al.*, 1980a,b; Kawamura *et al.*, 1981). A list of cloned *spo* and *ger* genes is presented in Table III. Although the exact functions of all but two of these genes are still unknown, the probable function of some of the genes has been identified by comparing the amino acid sequence homology of their gene products with that of known proteins.

The presence of some of the *spo* genes on high-copy plasmids inhibits sporulation. Recombinant plasmids carrying *spo0F* (Kawamura *et al.*, 1980a), *spoIIIG* (Ayaki and Kobayashi, 1984), *spoIVC* (Fujita and Kobayashi, 1985), *spoVE* (Yanada *et al.*, 1983), and *spoVG* (Banner *et al.*, 1983) inhibit sporulation of the wild-type strain, whereas those carrying *spo0B* (Hirochika *et al.*, 1981) do not have any effect on sporulation. Thus it is possible that these genes could titrate limiting cellular components (minor RNA polymerase holoenzymes or regulatory proteins) or produce proteins which are detrimental to sporulation at higher concentrations.

5.1. *spo0* Genes

Of the 10 known *spo0* loci (Piggot and Hoch, 1985) *spo0A*, *spo0B*, *spo0F*, and *spo0H* have been cloned and sequenced. Most of the *spo0* loci appear to code for one protein.

5.1.1. *spo0A*

In the hierarchy of *spo0* mutations, *spo0A* exerts the greatest pleiotropic effect and is believed to be of major importance (Piggot and Coote, 1976). The gene has been cloned (Ikeuchi *et al.*, 1983; Ferrari *et al.*, 1984) and codes for a protein with a molecular mass of 27,500 according to Kudoh *et al.* (1984) or 29,691 according to

Table III. Cloned Sporulation and Germination Genes

Gene	Molecular masses of product	References
<i>spo0A</i>	29,691	Ferrari <i>et al.</i> (1985b) Kudoh <i>et al.</i> (1985) Ikeuchi <i>et al.</i> (1983) Bouvier <i>et al.</i> (1984) Bonamy and Szulmajster (1982) Ferrari <i>et al.</i> (1982) Hirochika <i>et al.</i> (1981) Ferrari <i>et al.</i> (1985a) Ikeuchi <i>et al.</i> (1983) Ferrari <i>et al.</i> (1985b) Trach <i>et al.</i> (1985) Weir <i>et al.</i> (1984) Dubnau <i>et al.</i> (1987) Ramakrishna <i>et al.</i> (1984) Dubnau <i>et al.</i> (1987) Savva and Mandelstam (1984) Fort and Piggot (1984) Fort and Piggot (1984) Fort and Piggot (1984) Anaguchi <i>et al.</i> (1984) Stragier <i>et al.</i> (1984) Trempey <i>et al.</i> (1985b) Ayaki and Kobayashi (1984) Jenkinson and Mandelstam (1983) Butler and Mandelstam (1987) Fujita and Kobayashi (1985) Fort and Errington (1985) Fort and Errington (1985) Fort and Errington (1985) Fort and Errington (1985) Fort and Errington (1985) Yamada <i>et al.</i> (1983) Piggot <i>et al.</i> (1986) Segall and Losick (1977) Connors and Setlow (1985) Curiel-Quesada <i>et al.</i> (1983) Fliss and Setlow (1984a,b) Fliss <i>et al.</i> (1985)
<i>spo0B</i>	22,542	Moir (1981) Zuberi <i>et al.</i> (1987) Moir <i>et al.</i> (1985) Cutting and Mandelstam (1986) Warburg <i>et al.</i> (1985) Moir <i>et al.</i> (1985) Vasantha <i>et al.</i> (1983)
<i>spo0C^a</i>		
<i>spo0F</i>	14,286	
<i>spo0H</i> (<i>B. subtilis</i>)	30,000	
<i>spo0H</i> (<i>B. licheniformis</i>)	22,000	
<i>spoIIA</i>	13,100	
<i>spoIIAA</i>	16,300	
<i>spoIIAB</i>	22,200	
<i>spoIIAC</i>		
<i>spoIIIC</i>		
<i>spoIIG</i>	27,652	
<i>spoIIB</i>		
<i>spoIIE</i>		
<i>spoIVC</i>	23,100	
<i>spoVAA</i>	15,200	
<i>spoVAB</i>	16,100	
<i>spoVAC</i>	36,000	
<i>spoVAD</i>	34,300	
<i>spoVAE</i>		
<i>spoVE</i>		
<i>spoVG</i>		
SASP 1 (<i>B. subtilis</i>)		
SASP C (<i>B. megaterium</i>)		
SASP C-1-C-5 (<i>B. megaterium</i>)		
<i>gerA</i>		
<i>gerB</i>		
<i>gerJ</i>		
<i>gerM</i>		
<i>gdt</i>		

^a*spo0C* is now known to be a locus within *spo0A* (Ferrari *et al.*, 1985b).

Ferrari *et al.* (1985b). Genetic experiments have indicated that *spo0C* is actually a mutation in the C terminus of the *spo0A* gene (Ferrari *et al.*, 1985b) and that the mutations *sof* (Kawamura and Saito, 1983) and *rut* (Sharrock *et al.*, 1984) are mutations in the N terminus of the *spo0A* gene (Hoch *et al.*, 1985). Although most mutations in the *spo0A* locus cause asporogeny, other mutations in the locus such as *sof* and *rut*, which are probably identical mutations (Hoch *et al.*, 1985), allow the cell to sporulate. These mutations affect the N-terminal domain of the *spo0A* gene product, which still allows it to function and to suppress other *spo0* functions. Mutations in other domains of the *spo0A* gene product, however, do not allow it to function for sporulation. Therefore the Spo0A phenotype depends on the site of mutation in the *spo0A* gene and the actual function of the *spo0A* is probably not specific for sporulation. The Spo0A phenotype may arise as a consequence of the general function of the *spo0A* gene product.

The postulated major role for *spo0A* in the initiation of sporulation is supported by its ability to suppress other *spo0* mutations, including even a deletion mutation of *spo0F* (Kawamura and Saito, 1983; Sharrock *et al.*, 1984). An altered Spo0A protein (Sof or Rvt protein) can overcome the requirements for the *spo0F*, *spo0E*, and *spo0B* gene products. These results suggest that the products of the *spo0B*, *spo0E*, and *spo0F* genes normally modulate the function of the *spo0A* product; however, this modulation is unnecessary when a *sof* or *rut* mutation is present which allows the mutant *spo0A* product to bypass the *spo0B*, *spo0E*, and *spo0F* functions (Hoch *et al.*, 1985).

The *spo0A* gene is expressed primarily during the exponential phase of growth and is not essential for growth (Ferrari *et al.*, 1985b). However, its function appears to be absolutely necessary for the initiation of sporulation. A comparison of amino acid sequences has revealed a partial homology between the Spo0A protein with the Spo0F protein, and the OmpR and SfrA products of *E. coli* (Trach *et al.*, 1985). The *ompR* gene product is a regulator of the expression of the *ompC* and *ompF* genes (Hall and Silhavy, 1981a), which code for outer membrane porin proteins of *E. coli*. The *ompB* gene product is a DNA-binding protein and may be a positive regulator of gene expression (Hall and Silhavy, 1981b).

Thus the Spo0A product may also regulate the expression of genes which affect membrane functions in *B. subtilis*. It has been postulated that the *spo0* genes are involved in some type of nutritional sensing of the environment (Ferrari *et al.*, 1985a; Doi *et al.*, 1985). This idea is supported by the finding of Doi *et al.* (1985) that the *sof* mutant also has a Crs (catabolite-resistant sporulation) phenotype (Takahashi, 1979). Thus *spo0A* may code for a regulatory protein which responds to some mechanism or part of an apparatus of the membrane that senses the availability of carbohydrates. The interaction between the Spo0A protein and the sensing device is altered in *sof* mutants and allows sporulation initiation even in the presence of excess glucose (Doi *et al.*, 1985).

5.1.2. *spo0B*

The *spo0B* gene has been cloned by a number of investigators (Hirochika *et al.*, 1981; Bonamy and Szulmajster, 1982; Ferrari *et al.*, 1982; Bouvier *et al.*, 1984) and codes for a protein with a molecular mass of about 22,500. The sequence analysis of

the *spo0B* locus indicates that *spo0B* is part of an operon which contains at least one more downstream gene (Ferrari *et al.*, 1985a).

5.1.3. *spo0C*

The *spo0C* gene has now been identified as a locus in the *spo0A* gene (Ferrari *et al.*, 1985b). The *spo0C* mutation is located in the tenth codon from the C terminus of the *spo0A* gene and gives rise to a missense Spo0A protein which is partially active and results in a phenotype which is less pleiotropic than mutations in other sites of the gene. The *spo0C* gene was cloned along with *spo0A* in a temperate bacteriophage of *B. subtilis* (Ikeuchi *et al.*, 1983).

5.1.4. *spo0F*

The *spo0F* gene codes for a protein of 14,286 Da (Trach *et al.*, 1985, 1986). The derived amino acid sequence of the Spo0F protein shows homology with the N-terminal half of the protein coded by the *spo0A* gene and the OmpR and SfrA proteins of *E. coli* (Trach *et al.*, 1985). Since *ompR* controls the expression of *ompC* and *ompF* genes (see 5.1.1), *spo0F* like *spo0A* may regulate expression of genes which code for *B. subtilis* membrane components. Since membranes are involved in the sporulation process, the Spo phenotype of mutations in these genes may be an indirect consequence of damage to this regulatory system (Trach *et al.*, 1985).

The gene cloned initially by Shimotsu *et al.* (1983) as *spo0F* codes for a protein of 19,065 Da. This gene has now been located adjacent to the *spo0F* gene (Trach *et al.*, 1986; Yoshikawa *et al.*, 1986a,b). The *spo0F221* mutation (Yoshikawa *et al.*, 1986b) has been located to a codon for a leucine residue in the N terminus of the *spo0F* gene (Trach *et al.*, 1985).

The *spo0F* mutation can be suppressed by *sof* (suppressor of *spo0F*), which is actually a *spo0A* mutation (Kawamura and Saito, 1983; Sharrock *et al.*, 1984). Since even a deletion mutant of *spo0F* can be suppressed by *sof*, it indicates that the Spo0F protein normally modulates the activity of the Spo0A protein and that the latter plays a more direct role than the former in initiating sporulation.

5.1.5. *spo0H*

The *spo0H* gene has been cloned from *B. subtilis* (Weir *et al.*, 1984) and *B. licheniformis* (Ramakrishna *et al.*, 1984). The *B. subtilis* gene codes for a protein with a molecular mass of 30,000. Spo0H has amino acid homology with the major RNA polymerase σ^{43} factor and has been shown to be a minor σ factor (Dubnau *et al.*, 1987; Carter and Moran, 1987). This σ factor has been designated as σ^H and its gene as *sigH*.

5.1.6. *spo0K*

Although the *spo0K* gene has not been cloned to date, the mutual suppression between *rpoD47* (*crsA47*) and *spo0K* suggests that it may be a transcriptional regulatory factor (Kawamura *et al.*, 1985).

5.2. *spoII* Genes

Seven *spoII* loci control the developmental stages after forespore septum formation (Piggot and Hoch, 1985). The *spoIIA* (Sava and Mandelstam, 1984; Fort and Piggot, 1984), *spoIIC* (Anaguchi *et al.*, 1984), and *spoIIG* (Bonamy and Szulmajster, 1982; Ayaki and Kobayashi, 1984) genes have been cloned and sequenced. It was first noted by sequence analyses that the *spoIIG* gene product had a molecular mass of about 28,000 and a partial amino acid sequence homology with the *E. coli* σ^{70} factor (Stragier *et al.*, 1984) and the *B. subtilis* σ^{43} factor (Gitt *et al.*, 1985). Subsequent genetic studies by Trempy *et al.* (1985b) showed that *spoIIG* codes for the sporulation-specific σ^E factor (Trempy *et al.*, 1985c) of *B. subtilis* RNA polymerase (Haldenwang *et al.*, 1981). The σ^E factor is the first sporulation-specific σ factor which has been identified and mapped genetically. The protein is synthesized initially as a precursor with a molecular mass of 31,000 (Trempy *et al.*, 1985a; LaBell *et al.*, 1987) in the mother cell (Fukuda and Doi, 1977) and then is processed and transported to the forespore where it is found as a mature 29,000-Da protein (Nakayama *et al.*, 1980). It is possible that most genes controlled by σ^E promoters will be expressed in the forespore.

The recent sequence analysis of the *spoIIA* locus indicated that three genes were present in this site, coding for proteins with molecular masses of 13,100, 16,300, and 29,000 (Fort and Piggot, 1984). The protein product with a molecular mass of about 29,000 also has partial amino acid sequence homology (Errington *et al.*, 1985) with σ^{43} factor of *B. subtilis* (Gitt *et al.*, 1985). Thus it too is potentially a sporulation-specific factor, although more biochemical and genetic evidence is required for substantiation of this hypothesis.

The synthesis of sporulation-specific σ factors at stage II indicates that the RNA polymerase at this point is able to transcribe a number of sporulation genes, although no products have been identified as yet from σ^E -controlled genes. It is possible that sporulation-associated genes are also controlled by σ^E , since several intracellular and extracellular enzymes are synthesized at this time.

5.3. *spoIII* Genes

The *spoIII* genes control development of the forespore after engulfment has occurred. Six loci have been reported for these genes (Piggot and Hoch, 1985). The *spoIIIB* gene has been cloned in phage $\phi 105$ (Jenkinson and Mandelstam, 1983).

In an approach to determine whether a gene was expressed in the mother cell or the forespore, transformation of *spo* mutants which were blocked at or after stage III was examined (DeLencastre and Piggot, 1979). If transformation of *Spo*⁻ strains yielded *Spo*⁻ spores, then it was assumed that the site for expression of the *spo* gene was on the mother cell chromosome, i.e., the genome of the mother cell during sporulation had been transformed to *Spo*⁺, but not that of the forespore. Also it was assumed that transformation of only the mother cell genome was sufficient for sporulation to occur. Since the mother cell is destroyed upon release of the mature spore, the nontransformed spore genome would still be *Spo*⁻.

From these studies it was concluded that *spoIIID*, *spoIVA*, *spoVB*, and *spoVE* were expressed in the mother cell. For one locus, *spoVA*, it was assumed that it had

to be expressed in the forespore, since greater than 99% of the resulting spores were *Spo*⁺.

5.4. *spoIV* Genes

The seven *spoIV* loci are expressed during cortex formation. The *spoIVC* gene has been cloned by the prophage transformation method using phage $\phi 105$ (Fujita and Kobayashi, 1985). The *spoIVC* (Dancer and Mandelstam, 1981) and *spoIVF* (Lamont, 1984) loci both contain at least two genes.

Although *SpoIV*⁻ cells develop normally until stage IV, an analysis of their protein synthesis patterns reveals that they differ from the wild type as early as T₂ (i.e., stage II) (Boschwitz and Yudkin, 1983). Thus these and other (Jenkinson *et al.*, 1981) results cast some doubt on the classification of mutants, since it might be assumed that stage IV mutants follow a normal pattern of development up to stage IV.

By transformation experiments described in Section 5.3, it was determined that *spoIVA* was expressed from the mother cell genome rather than the forespore genome (DeLencastre and Piggot, 1979).

5.5. *spoV* Genes

Many of the nine *spoV* loci are involved in the assembly of the spore coat proteins on the forespore. The *spoVA* locus has been sequenced and it contains five open reading frames coding for proteins of molecular masses of 15,000–36,000 (Fort and Errington, 1985). It is the largest polycistronic sporulation operon yet characterized. Although the functions of these *SpoV* proteins are not known, they are highly basic and hydrophobic, which suggests that they may have some relationship to SASP, membrane, or DNA-binding proteins.

spoVA is expressed in the forespore whereas *spoVB* is expressed in the mother cell (DeLencastre and Piggot, 1979). *spoVG*, previously called the 0.4-kilobase gene (Haldenwang *et al.*, 1980), maps near *tms26* at 6° (Piggot and Hoch, 1985).

The interpretation of *spoV* mutants is complicated by the fact that the formation of the spore coat in stage V involves the deposition of coat proteins which had been synthesized during stages II and III (Jenkinson *et al.*, 1981). Also the *spoVG* gene is transcribed within 30 min of the start of sporulation (Segall and Losick, 1977). Thus it is highly likely that mutations in genes expressed early in sporulation are resulting in a stage V phenotype.

5.6. *spoVI* Genes

The two *spoVI* loci are involved in the maturation of the spore. The spores produced by *spoVIA* mutants germinate slowly and are sensitive to lysozyme (Jenkinson, 1981). Their spore coats lack a 36,000-Da protein which is normally deposited during stage V. This locus is different from the *ger-36* mutant which maps close by (Moir *et al.*, 1979).

Another locus, *spoVIB*, is involved in the proper assembly of coat proteins

(Jenkinson, 1983). The assembly of the coat is delayed in *spoVIB* mutants and misarrangement of the surface proteins, particularly a 12,000-Da protein, is evident. These spores are very sensitive to lysozyme and sporulate and germinate more slowly than wild-type cells.

5.7. *ger* Genes

Mutations in the 13 *ger* loci cause defective germination of spores in a range of germinants (Moir *et al.*, 1985). More than 100 mutants of *B. subtilis* have been isolated whose spores germinate abnormally. Although several of the *ger* genes have been cloned, they have not as yet been thoroughly characterized.

5.7.1. *gerA* Genes

The *gerA* mutants are altered in their response to alanine and related amino acid germinants, but germinated normally in a mixture of glucose, fructose, asparagine, and KCl (Moir *et al.*, 1985). The *gerA* locus, which maps close to *citG*, may encode a germination receptor common to L-alanine and its analogs. Three *gerA* complementation groups have been identified (Zuberi *et al.*, 1985), and sequence studies revealed the presence of three genes that code for polypeptides with masses of 53,506, 41,257, and 42,363 Da (Zuberi *et al.*, 1987).

5.7.2. *gerD* Gene

The *gerD* gene is located close to *rpoB* and *rpsE* (Moir *et al.*, 1979). Germination in L-alanine or in a mixture of L-alanine, glucose, fructose, and KCl was slower than that of the wild type. Plasmids carrying DNA from the *gerD* region have been isolated (Moir *et al.*, 1985).

5.7.3. *gerE* Gene

The *gerE* gene maps near *uwzB* and *spoVIA*. The altered germination of these spores may be due to an alteration in a spore coat protein (Moir, 1981). The *gerE* region has been cloned and sequenced and codes for a small protein of 74 amino acid residues (Cutting and Mandelstam, 1986).

5.7.4. *gerJ* Gene

The *gerJ* locus is located close to *aroC* and *ser-22*. *gerJ* mutants form phase bright spores, which respond to the same germinants as wild type, but during germination they reach only a phase grey instead of a phase dark stage. Their spores also acquire resistance to heat and organic chemicals more slowly than the wild type and the spores are more sensitive to heating at 90°C. Their spores may have an altered cortex structure perhaps caused by a defect in an enzyme involved in cortex synthesis (Warburg *et al.*, 1985).

5.7.5. *gerM* Gene

The *gerM* is linked to *citF* and *itvB*. The *gerM96* mutant forms lysozyme-resistant spores which germinate only to an intermediate stage in L-alanine or in a mixture of glucose, fructose, L-asparagine, and KCl. The loss of heat resistance was normal, but the germinating spores only achieved a phase grey state. Plasmids carrying the *gerM96* gene were isolated (Moir *et al.*, 1985) by the rescue vector method of Youngman *et al.* (1985) (see Section 4.1 and Chapter 6, Section 7.4).

6. TRANSCRIPTIONAL REGULATION DURING SPORULATION

Transcriptional regulation during sporulation has been demonstrated by the appearance of new classes of mRNA (Doi and Igarashi, 1964; Yamakawa and Doi, 1971; Sumida-Yasumoto and Doi, 1977) and of sporulation-associated and sporulation-specific proteins (see Section 3). This differential expression of sporulation genes is controlled by a complex transcription mechanism consisting of an array of RNA polymerase σ factors (Losick and Pero, 1981; Doi, 1982a,b; Doi and Wang, 1986), each allowing the temporal recognition of different sets of promoters (Moran *et al.*, 1981a,b, 1982; Johnson *et al.*, 1983), and the activity of other transcription regulatory factors which have not been characterized to date.

6.1. Multiple RNA Polymerase Holoenzymes

The presence of multiple RNA polymerase holoenzymes in *B. subtilis* has been well documented. In the vegetative cell there are at least four RNA polymerase holoenzymes which differ by virtue of having different σ factors associated with a common core enzyme (Table IV). The major form of the enzyme is designated as $E\sigma^{43}$ or $E\sigma^A$ (E = core enzyme; the number indicates the molecular mass of the σ factor $\times 10^{-3}$; the superscript is also used to describe a particular σ factor). This enzyme form was previously designated as $E\sigma^{55}$ but recent sequence analysis of the major σ factor gene showed that the molecular mass was closer to 43,000 (Gitt *et al.*, 1985). $E\sigma^{43}$ comprises about 90–95% of the RNA polymerase present in vegetative cells (Doi *et al.*, 1980). The minor forms of vegetative RNA polymerase contain σ factors with molecular masses of 37,000 (σ^{37} or σ^B) (Haldenwang and Losick, 1980), 32,000 (σ^{32} or σ^C) (Johnson *et al.*, 1983), 30,000 (σ^H) (Dubnau *et al.*, 1987; Carter and Moran, 1987), and 28,000 (σ^{28} or σ^D) (Wiggs *et al.*, 1981), and these forms compose about 5–10% of the total enzyme. The promoter specificities of these holoenzymes are listed in Table V.

$E\sigma^B$ is most active near the end of the exponential phase of growth (Losick, 1982). The *sigB* gene has been cloned and sequenced (Binnie *et al.*, 1986; Duncan *et al.*, 1987). $E\sigma^D$ appears to have an activity pattern similar to that of $E\sigma^B$. $E\sigma^D$ is active during growth, but its activity drops severely at the end of exponential growth (Gilman and Chamberlin, 1983).

$E\sigma^{43}$ plays a major role in transcription during growth, but its activity during sporulation is still not certain (Linn *et al.*, 1973). Although it can be found in sporulating cells (Tjian and Losick, 1974; Fukuda and Doi, 1977), it has not been

Table IV. RNA Polymerase Holoenzyme Forms in *B. subtilis*

Stage of Growth	RNA polymerase	References
Vegetative	E σ^{13a} (E σ^A)	Shorenstein and Losick (1973) Fukuda <i>et al.</i> (1975) Gitt <i>et al.</i> (1985)
	E σ^B	Haldenwang and Losick (1980) Duncan <i>et al.</i> (1987) Binnie <i>et al.</i> (1986) Igo <i>et al.</i> (1987)
	E σ^C	Johnson <i>et al.</i> (1983)
	E σ^D	Wiggs <i>et al.</i> (1981)
	E σ^{13}	Tjian and Losick (1974) Doi <i>et al.</i> (1983)
Stage 0	E σ^B	Haldenwang and Losick (1980)
	E σ^D	Johnson <i>et al.</i> (1983)
	E σ^{13}	Gilman and Chamberlin (1983) Tjian and Losick (1974) Doi <i>et al.</i> (1980)
	E σ^B	Johnson <i>et al.</i> (1983)
	E σ^C	Johnson <i>et al.</i> (1983)
Stage II	E σ^E	Fukuda <i>et al.</i> (1975) Linn <i>et al.</i> (1975) Fukuda and Doi (1977) Nakayama <i>et al.</i> (1978) Haldenwang <i>et al.</i> (1981) Stragier <i>et al.</i> (1984)
	E σ^{13}	Trempey <i>et al.</i> (1985bc) Gilman and Chamberlin (1983) Fort and Piggot (1984) Errington <i>et al.</i> (1985) Tjian and Losick (1974) Doi <i>et al.</i> (1980)
	E σ^B	Johnson <i>et al.</i> (1983)
	E σ^C	Johnson <i>et al.</i> (1983)
	E σ^E	Fukuda <i>et al.</i> (1975) Linn <i>et al.</i> (1975)
Stage III	E σ^{13}	Nakayama <i>et al.</i> (1978) Haldenwang <i>et al.</i> (1981) Stragier <i>et al.</i> (1984) Trempey <i>et al.</i> (1985bc) Fort and Piggot (1984) Errington <i>et al.</i> (1985)
	E σ^B	Johnson <i>et al.</i> (1983)
	E σ^C	Johnson <i>et al.</i> (1983)
	E σ^E	Fukuda <i>et al.</i> (1975) Linn <i>et al.</i> (1975)
	E σ^{13}	Nakayama <i>et al.</i> (1978) Haldenwang <i>et al.</i> (1981) Stragier <i>et al.</i> (1984) Trempey <i>et al.</i> (1985bc) Fort and Piggot (1984) Errington <i>et al.</i> (1985)

^aE σ^{13} (E σ^A) was previously designated as E σ^{55} based on its electrophoretic mobility. The sequence analysis indicates that σ^A has a molecular weight of around 43,000 (Gitt *et al.*, 1985).

^bTentatively identified as a σ factor by sequence similarity to σ^{43} (Errington *et al.*, 1985).

Table V. Promoter Sequences for Various Holoenzymes^a

Holoenzyme	-35	-10	References
<i>B. subtilis</i>			
E σ^A (E σ^{43})	TTGACA	N ₁₇ TATAAT	Moran <i>et al.</i> (1982)
E σ^B	N.D.	N.D.	
E σ^C	N.D.	N.D.	
E σ^D	CTAAA	N ₁₆ CCGATAT	Gilman <i>et al.</i> (1981)
E σ^E	GAANAANT	CATATTNT	Rather <i>et al.</i> (1986)
E σ^{11}	GCAGGANTT	GAATTNTT	Carter <i>et al.</i> (1988)

^aN, any base; N.D., not determined.

demonstrated to function actively during spore formation. The subtilisin gene (*aprA*) appears to be regulated by a σ^{43} promoter which can be utilized as late as T₅ (Park and Doi, unpublished results). Thus σ^{43} enzyme may be active even during late sporulation.

The E σ^D may recognize heat shock protein genes in *B. subtilis*, since a σ^D promoter is found to precede the *rpoD* gene in the σ^{43} operon (Wang *et al.*, 1985; Wang and Doi, 1986a) and the *rpoD* gene responds to heat shock treatment (Wang and Doi, 1986b). However, not all heat shock promoters are σ^D promoters (Gilman and Chamberlin, 1983), since the heat shock response still occurred in *spoD* mutants which no longer made transcripts from σ^D -controlled promoters (Kawamura and Doi, unpublished data). Recent data indicate that E σ^D controls the expression of flagella and chemotaxis genes (Helmann and Chamberlin, 1987).

Since the minor RNA polymerase enzymes found in vegetative cells have been found to be active under stress, e.g., nutritional deprivation and heat shock, and the minor σ enzyme (σ^{32}) in *E. coli* (Grossman *et al.*, 1984) also responds to heat shock situations, it appears that minor enzymes may have evolved to control the expression of stress-related genes of the cell. These could include genes which respond to nutritional deprivation, heat shock, ultraviolet light irradiation, toxic chemicals, genetic invasion by DNA or phage genomes.

6.2. Temporally Regulated Promoters

Two types of temporally regulated promoters have been found to date; these control the expression of genes after the end of the logarithmic phase of growth. One type controls the expression of genes which are not essential for sporulation and the other presumably controls genes essential for normal sporulation.

The *spoVG* and *cic* genes contain overlapping σ^B and σ^C promoters (Johnson *et al.*, 1983; Stephens *et al.*, 1984a). The *spoVG* is required for normal development of the spore coat and thus it is a sporulation-specific gene controlled in a temporal fashion. The interesting point about this gene is that it is expressed very early in sporulation, at about T_{0.5}, but it regulates the morphological development of the spore at about T₅ (Segall and Losick, 1977). Other temporally regulated promoters have been isolated including σ^B and σ^E promoters (Haldenwang *et al.*, 1981; Wang and Doi, 1984).

Why do many *B. subtilis* genes have more than one promoter? *Bacillus subtilis* is transcriptionally active during growth and the sporulation phase. The analyses of mRNA populations revealed that most of the genes expressed during growth were still expressed during sporulation (Sumida-Yasumoto and Doi, 1977). Since σ^{43} enzyme is utilized during growth and minor σ enzymes are more active during sporulation, it is possible that multiple promoters have evolved so that housekeeping genes are capable of being expressed at all stages of growth.

The presence of multiple promoters for sporulation genes suggests that these genes are expressed over extended periods of the sporulation phase and therefore require promoters which might be expressed during early and late sporulation. Presumably those genes expressed only at late sporulation stages may have only one promoter. Analyses of more promoter regions should reveal whether this is the case.

6.3. σ^E Enzyme

The product of *spoIIIG* gene, σ^E , is synthesized during early sporulation. The sporulation-specific $E\sigma^E$ is necessary for transcription of developmental genes after forespore septum formation. σ^E is one of only two known *spo* gene products (the other being *spoOH* product or SigH) which have been identified to date (presumably SASPs and spore coat proteins are also *spo* genes). σ^E has partial amino acid sequence homology with σ^{43} (Stragier *et al.*, 1984; Gitt *et al.*, 1985).

One of the products of the *spoIIA* locus has a molecular mass of 29,000 (P29) (Fort and Piggot, 1984) and also has partial amino acid sequence homology (Erington *et al.*, 1985) with σ^{43} (Gitt *et al.*, 1985). Thus the occurrence of sporulation σ factors indicates that a sequential expression of σ factors partially controls the temporally regulated expression of sporulation genes as suggested by Losick and Pero (1981). This is supported by the fact that each σ factor controls the recognition of specific promoters (Losick and Pero, 1981; Doi, 1982a; Doi and Wang, 1986). Thus each σ factor regulates the expression of a set of genes containing similar promoter sequences. It is likely that other σ factors will be found for the sporulation phase.

6.4. RNA Polymerase, *spo0* Genes, and Catabolite Repression

A relationship between RNA polymerase activity, *spo0* gene products, and catabolite repression has been observed. The activity of $E\sigma^D$ is regulated by the products from *spo0A*, *spo0B*, *spo0E*, and *spo0F* genes (Gilman and Chamberlin, 1983). The activity of $E\sigma^B$ is regulated by the products from *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* genes (Ollington *et al.*, 1981). These results indicate that the products of *spo0* genes are involved either directly or indirectly in transcription by these minor RNA polymerase forms both during growth (σ^D) and during early sporulation (σ^B).

This relationship has been extended even further by the observation that a mutation, *rpoD47* (Price and Doi, 1985; Kawamura *et al.*, 1985), in the major σ^{43} gene (Price *et al.*, 1983) can suppress mutations in *spo0B*, *spo0D*, *spo0F*, *spo0J*, and *spo0K*, but not *spo0A* (Doi *et al.*, 1985; Kawamura *et al.*, 1985; Leung *et al.*, 1985).

The *rpoD47* mutation, initially selected as *crsA47* (catabolite-resistant sporulation) by Takahashi (1979), allows *B. subtilis* cells to sporulate in the presence of high levels of glucose. This and other *crs* mutants were mapped to six loci (*crsA* to *crsF*) on the *B. subtilis* map. The *crsE* locus is within the RNA polymerase *rpoB-rpoC* ($\beta\beta'$) region (Sun and Takahashi, 1984) and the *crsC* locus was mapped close to *spo0A* (Sun and Takahashi, 1982). It appears that *crsC* actually is a mutation in *spo0A* (F. Kawamura, personal communication). This observation is supported by the fact that *sof*, which is a mutation in *spo0A* (Hoch *et al.*, 1985), also displays a *Crs* phenotype (Doi *et al.*, 1985). It is therefore possible that *crsC* is identical to *sof* or is a mutation very close to it in *spo0A*. Thus mutations in either *rpoD* or *spo0A* result in a catabolite-resistant sporulation. Furthermore *spo0A* can suppress *rpoD47* (*crsA47*) (Kawamura *et al.*, 1985).

Molecular mapping experiments localized the *rpoD47* mutation to a proline codon (CCT) which had been changed to a phenylalanine codon (TTT) at amino acid residue 290 in the σ^{43} gene (*rpoD*) (Price and Doi, 1985; Kawamura *et al.*, 1985).

Subsequently, a number of suppressor mutations for *rpoD47* were selected. The suppression of *rpoD47* could be readily detected on minimal glucose plates, since *rpoD47* strains produced small colonies and suppressed strains produced large colonies (Sun and Takahashi, 1985). Among the many suppressor mutations obtained, mutations in *spo0A*, *spo0D*, *spo0F*, and *spo0K* were found to be effective suppressors (Kawamura *et al.*, 1985). Thus the σ^{43} factor interacts either directly or indirectly with the products of a number of *spo0* genes. This in turn suggests that the major σ^{43} plays a major role in determining whether a cell will initiate a number of cellular responses to nutrient deficiency. For instance, the chemotaxis response including flagella formation, catabolite derepression (including internal metabolic reorganization and extracellular enzyme synthesis), asymmetrical membrane synthesis, and initiation of sporulation may depend on the interaction of the σ^{43} and the other nutrient sensing Spo0 proteins.

The Spo⁻ phenotype may be the result of mutations in transcriptional regulatory factors (i.e., *spo0* genes) which affect the transcription of genes involved in various membrane functions. Not all of the *spo0* functions are required for growth, but they may be necessary for sensing and responding to nutrient deprivation and for forming the special asymmetrical forespore membrane. The major role of σ^{43} and the Spo0A protein in nutrient sensing and catabolite repression is indicated by the *Crs* phenotype, which results from mutations in either of their genes.

7. REGULATORY EVENTS IN INITIATING SPORULATION

7.1. Replicative Activation of Sporulation

When cells were transferred from a rich medium to a sporulation resuspension medium which exerted nutritional stress on the cells (Sterlini and Mandelstam, 1969), sporulation was initiated only after a final round of DNA replication had been initiated (Mandelstam and Higgs, 1974). By tests at various times during synchronous DNA replication, it was determined that initiation of sporulation oc-

curred optimally when the DNA had replicated for about 15 min. This brought the replicating fork to the *cysA-sul^r* region of the chromosome. It was postulated that replication of the DNA molecule near a *spo0* gene would allow transcription to be initiated from this gene, which in turn triggered the whole sporulation response. The mechanism of transcriptional activation of sporulation during replication remains a hypothesis. No concrete evidence for this proposition has been found to date; however, *spo0H* and *spo0J* genes are in the regions of the *B. subtilis* chromosome when optimal time and bidirectionality of DNA replication are considered.

Since release from catabolite repression has also been postulated as a mechanism for initiating sporulation (Schaeffer *et al.*, 1965b), the relationship between catabolite-repressed enzymes and replication-activated sporulation was examined. It was found that although sporulation remained repressed unless chromosome replication was allowed to proceed, the derepression of histidase, sucrase, and α -glucosidase proceeded normally in the absence of DNA synthesis (Coote, 1974). It was thus possible to separate catabolite repression from replication-activated sporulation initiation, since catabolite derepression was not dependent on the replication phase of the chromosome (Coote, 1974). Furthermore, one of the earliest marker events in sporulation, the biosynthesis of the extracellular alkaline protease, is not related to the state of chromosome replication (Clarke and Mandelstam, 1980).

Catabolite repression, alkaline serine protease induction, and the repression of sporulation may be controlled by a common overall regulator, since they are initiated only after certain nutrients are depleted; however, the specific regulatory mechanisms for these phenomena are quite different as far as the requirement for DNA replication is concerned.

7.2. Role of Guanine Nucleotides

Although cells will not normally initiate sporulation or sporulate in the presence of rapidly metabolizable carbon, nitrogen, or phosphorus sources, the partial inhibition of the synthesis of GTP will cause massive sporulation even in the presence of favorable nutritional compounds (Freese *et al.*, 1979; Freese, 1981). Thus the effect of compounds which ordinarily cause catabolite repression of sporulation can be bypassed by conditions which reduce the GTP concentration of the cell. These conditions include the use of leaky purine-requiring auxotrophs (Freese *et al.*, 1978); inhibitors of purine biosynthesis, particularly of GTP (Mitani *et al.*, 1977; Heinze *et al.*, 1978; Lopez *et al.*, 1979); and stringent conditions of growth which increase ppGpp concentrations but which reduce GTP levels (Lopez *et al.*, 1981a,b). The stringent response is not in itself necessary for sporulation, since *relA* strains can sporulate in nutrient sporulation medium (Nishino *et al.*, 1979) or in synthetic medium containing decoyne, an inhibitor of GMP synthetase. Decoyne decreases the synthesis of guanine nucleotides but does not produce a stringent response (Lopez *et al.*, 1981b).

Catabolite repression does not prevent sporulation directly, since the expression of catabolite-repressed genes may be inhibited but sporulation can still be induced by the presence of decoyne (Lopez *et al.*, 1980).

All nutritional conditions which initiate sporulation have been correlated with a

decrease in GTP concentration of the cell, whereas other nucleotides have increased under some and decreased under other nutritional conditions (Freese, 1981).

A model has been proposed in which GTP either directly or indirectly serves as a corepressor to a repressor which binds to a methylated regulatory site on the DNA and prevents transcription of sporulation genes. When the GTP level is reduced, the repressor falls off allowing expression of the sporulation genes (Freese *et al.*, 1985).

Thus the initiation of sporulation could be a cascade of regulatory effects, each affecting several pathways of gene expression, but all leading ultimately to the control of expression of a key gene or set of genes.

7.3. Role of the Membrane

There is one common cellular location for a number of phenotypic aberrations in many early asporogenous strains, namely, the membrane. The major cytological effect of these mutations is the absence of asymmetrical forespore membrane formation, but there are a number of other effects such as lowered transformability (Schaeffer, 1969), absence of certain membrane proteins (Chui *et al.*, 1984), phenotypic reversion by polymyxin resistance (Guespin-Michel, 1971), bacteriophage tolerance (Ito, 1973), the absence of expression of extracellular enzyme genes (Hoch and Spizizen, 1969), the repression of chemotaxis and motility (Ordal *et al.*, 1985), and the possibility that *spoA* controls expression of membrane protein genes (Ferrari *et al.*, 1985b).

A more precise link has been established between the development of competence for transformation and sporulation (Sadaie and Kada, 1983a). Competent cell formation was blocked by 0.7 M ethanol, which is a specific inhibitor of early events of sporulation, including forespore septum formation. The development of competence was also affected by several *spo0* mutations. Mutations in *spo0A*, *spo0B*, *spo0D*, *spo0E*, *spo0F*, *spo0H*, and *spo0K* repressed whereas mutations in *spo0C* (now known to be located in *spo0A*), *spo0G*, and *spo0J* had little effect on the development of competence. These results suggest that common steps are involved both in development of competence and in forespore septum formation and that competent cells are formed from early sporulating cells.

In addition, several septum-initiation (*div*) mutants, which exhibit filamentous growth at 45°C, were found to be defective in competence development and sporulation (Sadaie and Kada, 1983b). It has been proposed from these studies that some of the initial steps of septation are shared in competence development and in forespore septum formation. However, these two processes are distinct from the septation mechanism observed in vegetative cell division.

The fact that some chemotaxis (*che*) mutants are also asporogenous (Ordal *et al.*, 1985) suggests that alterations of membrane-associated functions could indirectly affect sporulation.

All the evidence suggests that either the initiation of sporulation is related directly to membrane functions or the regulatory mechanism that controls membrane functions also controls functions for the initiation of sporulation. Since the nutritional sensors of the cell, including those for chemotaxis, are associated with

the cell membrane, and the catabolite repression mechanism is suggested to be linked to these sensors, a picture is emerging as to how the environmental signals may be relayed from the membrane to the genome (see Section 8).

8. SUMMARY AND OVERVIEW OF THE CONTROL OF INITIATION OF SPORULATION

From various experimental approaches it was demonstrated that initiation of sporulation is affected by the nutritional environment of the cell (catabolite repression), the state of DNA replication, the level of GTP in the cell, the activity of *spo0* genes, the function of RNA polymerase holoenzymes, and the regulators of membrane genes and functions. Although all of these factors affect sporulation, some of the responses appear to be closer to the sporulation process than others.

The initiation of sporulation is controlled at various regulatory levels in the cell. Since sporulation is the final cellular response to nutrient deprivation, it is preceded by a number of cellular activities which attempt to alleviate the stressful situation prior to committing the cell to a state of dormancy.

The early responses include those mechanisms which attempt to overcome a deficiency in the nutrient supply for the cell. This includes the mobilization of the chemotaxis apparatus to "search" for more nutrients. This is accompanied by flagella synthesis, increased motility, synthesis of a number of intracellular enzymes which can utilize the byproducts of growth and the synthesis of extracellular enzymes which can provide monomers from environmental biopolymers. If these responses do not improve the nutritional state of the cell, then sporulation is initiated. These responses could all be controlled by the mechanism of catabolite repression, which could be considered as part of the sensory device of the cell.

There is another link between chemotaxis and sporulation, since some *che* mutants are asporogenous. Is it possible that some *spo0* mutants are actually *che* mutants and that the whole sensing apparatus includes both types of functions? A systematic analysis of the *spo0* mutants for their chemotactic response may reveal this relationship. It is possible that some of the *che* and *spo0* genes code for transcription regulatory factors which control the expression of genes required for the sensing apparatus. If one considers catabolite repression as part of the sensory device, there appears to be a link between catabolite repression, *Spo0* functions, *Che* functions, and RNA polymerase.

The initiation of sporulation and the other responses to nutrient deprivation are controlled by the direct or indirect action of *spo0* genes with the transcription machinery including the major RNA polymerase enzyme, *Eσ⁴³*, and the minor *σ* enzymes. The *spo0* gene products are present in vegetative cells but are not necessary for growth. They appear to sense the availability of nutrients to the cell. When nutrients become depleted, these *Spo0* products respond by interacting (directly or indirectly) with the RNA polymerase holoenzymes to trigger the expression of genes involved in metabolism (catabolite derepression of intracellular enzymes) and membrane functions such as motility (flagella formation), extracellular enzymes (hydrolytic enzymes), and sporulation genes (asymmetrical forespore membrane formation).

GTP (or other guanine nucleotide derivatives) interacts with either *Spo0* products, RNA polymerase, or other regulatory proteins and acts as a coeffector in gene repression or derepression. This level of activity is more directly associated with the expression of sporulation genes than with catabolite-repressed genes, since the reduction of intracellular levels of GTP can result in sporulation in the absence of catabolite derepression of genes.

A mutation in a *spo0* gene reduces the cell's ability to respond to environmental changes and has a pleiotropic effect, since the cascade of membrane-controlled functions is not initiated. However, some of the *spo0* mutations can be compensated by a mutation (*spoD47*) in either the *σ⁴³* gene or in *spo0A*, indicating that interactions are occurring between the products of these two genes and the other *spo0* genes. The mutations in *spo0* genes are also suppressed by a mutation in *spo0A* (*sof*). Thus *σ⁴³* and *Spo0A* protein carry out major regulatory roles for the initiation of nutrient stress responses and both functions are essential for this purpose. Mutations in either gene can result in the *Crs* phenotype, indicating a functional link between *σ⁴³* and the *Spo0A* protein. It is interesting that mutations in *spo0A* cannot be suppressed by the *spoD47* (*crsA*) mutation, although the reverse is true.

A number of phenomena associated with the initiation of sporulation have been shown to be in separate functional pathways (see Fig. 3). Catabolite derepression of a number of genes is the major manifestation of carbohydrate and nitrogen depletion of the medium. The control of catabolite repression and the expression of the subtilisin gene have been dissociated from the specific pathway of sporulation; sporulation can be initiated while catabolite-repressed genes are still repressed

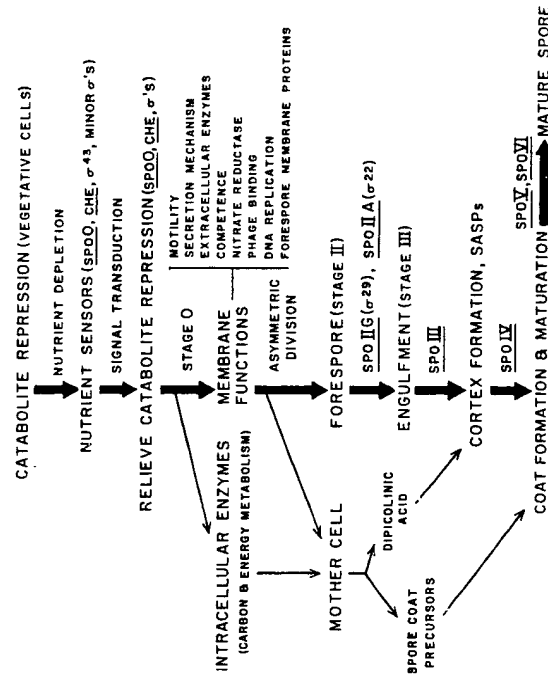


Figure 3. A summary of some of the events and proposed regulatory mechanisms during initiation and the various stages of sporulation in *B. subtilis*. See text for the discussion of the proposed control elements of sporulating *Bacillus*.

and when the subtilisin gene has been eliminated from the chromosome by deletion. Furthermore, while the initiation of sporulation requires a particular stage of chromosome replication, catabolite-repressed genes can be expressed at any stage of chromosome replication. Although aberrations in the overall regulatory function can affect these multiple pathways simultaneously, the specific functions required for the sporulation process can be delineated from those of others.

A much better understanding of the roles and relationships among catabolite repression, chemotactic responses, membrane functions, *spo0* genes and their products, and RNA polymerase and its transcription factors in the initiation process of sporulation will be obtained in the near future. In addition, the identification of many more sporulation and germination genes and their protein products appears imminent.

One of the possible practical uses that might be derived from the studies on the transcription system of sporulating cells is the utilization of promoters for the expression of foreign genes in *B. subtilis* during the stationary phase. If these promoters are also fused to signal peptide sequences and foreign genes, it may be possible to synthesize and secrete foreign proteins into the medium during sporulation (Doi and Wang 1986).

Thus the application of recombinant DNA technology has provided much novel information concerning sporulation and has opened a new vista on the roles of various interrelated factors during the initiation of sporulation. A more precise definition of the sporulation genes and their mechanisms of action should soon be forthcoming.

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MicroReview

Pulling the trigger: the mechanism of bacterial spore germination

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Summary

In spite of displaying the most extreme dormancy and resistance properties known among living systems, bacterial endospores retain an alert environment-sensing mechanism that can respond within seconds to the presence of specific germinants. This germination response is triggered in the absence of both germinant and germinant-stimulated metabolism. Genes coding for components of the sensing mechanism in spores of *Bacillus subtilis* have been cloned and sequenced. However, the molecular mechanism whereby these receptors interact with germinants to initiate the germination response is unknown. Recent evidence has suggested that in spores of *Bacillus megaterium* KM, proteolytic activation of an autolytic enzyme constitutes part of the germination trigger reaction.

Environmental sensing and spore dormancy

Microbial cells possess environment-sensing mechanisms that respond to a variety of stimuli, including chemotactic gradients, heat shock, changes in nutrient status and the presence of deleterious chemicals. Many of these mechanisms have been recognized to include a two-component protein system with conserved functional domains (Ronson *et al.*, 1987; Kofoed and Parkinson, 1988). Such responses are dependent on metabolic signalling events within the cell and some (e.g. sporulation and the heat-shock response) require the transcription of new sets of genes mediated, at least in part, by alternative sigma factors. Bacterial endospores, produced as a result of differentiation of *Bacillus* and *Clostridium* species, possess a different and possibly unique class of environment-sensing mechanism. This mechanism, essential for the germination response, is the subject of this review.

The intrinsic resistance, dormancy and germination properties of bacterial spores are dependent on an integral spore structure (Fig. 1) progressively assembled during sporulation and which comprises several spore-specific components (Ellar, 1978). The inner-most compartment, the spore core, contains the cellular components (e.g. DNA, RNA and metabolic enzymes) necessary for establishment of a vegetative cell cytoplasm following spore germination and outgrowth. Surrounding the core is the spore inner membrane, a lipid bilayer with no detectable fluidity, presumably as a result of polycrystalline structure (Stewart *et al.*, 1980). The spore cortex and primordial cell wall comprise a thick layer of peptidoglycan which forms a cage-like structure around the core and inner membrane (Warth, 1978). The remainder of the outer membrane surrounds the spore cortex which is, in turn, enclosed by the complex proteinaceous spore coats. Spores of several, but not all, species are finally enveloped by an exosporium.

Bacterial spores exhibit no detectable metabolism and can withstand extremes of pH, temperature, desiccation, humidity and radiation which would rapidly kill the vegetative cell from which they arose. These remarkable spore properties are dependent on the dormant spore structure and are the cumulative effects of several independent mechanisms. The spore core is dehydrated and its components immobilized in a lattice of salts of divalent metal ions and dipicolinic acid, which confers metabolic dormancy and heat resistance to core components (Gould, 1983). In addition, u.v. resistance is established by complexing spore DNA with spore-specific, acid-soluble, low molecular-weight proteins (Setlow, 1988). Finally, the spore coats prevent access of deleterious chemicals and enzymes to the inner spore compartments.

Paradoxically, despite this extreme dormancy, spores retain an alert sensory mechanism which can, within seconds, respond to a favourable environmental stimulus and trigger the cascade of events comprising the germination response.

Germination

We define spore germination as a series of inter-related degradative events, triggered by specific germinants,

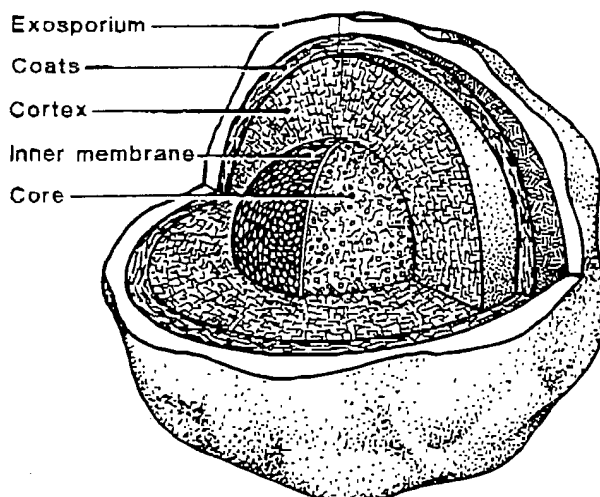


Fig. 1. Generalized structure of bacterial endospores.

which leads to the loss of typical dormant spore properties. Subsequently, outgrowth leads to the formation of a new vegetative cell. Prior activation increases the rate and extent of germination of a spore population and is conveniently achieved experimentally by sublethal heat treatment. The molecular basis of this process, which stimulates the germination response mechanism, is unknown.

Germination is initiated by interaction of the spore with specific germinants, the most common nutrient germinant being L-alanine. Initial interaction of the germinant with the spore constitutes the trigger reaction and irreversibly commits the spore to undergo the complex series of germination events (Stewart *et al.*, 1981). These triggering and commitment processes represent the primary environment-sensing mechanism and are considered in the rest of this review.

Germinant receptors and the trigger reaction

A major thrust of spore-germination research has inevitably been towards identification of the germinant receptor and elucidation of its function; this goal, however, remains to be achieved. In many species, L-alanine is the major germinant and D-alanine acts as a competitive inhibitor, binding at the same site as L-alanine (Stewart *et al.*, 1981). These stereospecific properties of the germinant receptor suggest that it must be a protein which is allosterically regulated. Although the nature of the germinant receptor is unknown, the following evidence suggests that it has an inner membrane location. Spores can be germinated after extraction of the spore coats and refractile spore protoplasts can be produced with no coats or cortex, which also exhibit an apparent germination

response (Fitz-James, 1971; Vary, 1973). A role for the spore coats in germination is, however, suggested by the observation that spores of *gerE* mutants of *Bacillus subtilis*, which have an altered coat structure, also exhibit abnormal germination characteristics (Moir, 1981). For spores of *Bacillus megaterium* QMB1551, one of the major germinants is L-proline. An affinity analogue of L-proline (L-proline chloromethylketone: PCK) and the germination inhibitor acetic anhydride both label a 10.2 kD inner-membrane protein (Racine *et al.*, 1981; Rossignol and Vary, 1979). In addition, L-proline causes fluidity and anisotropy changes in isolated spore membranes (Skomurski *et al.*, 1983). PCK labelling was diminished and anisotropy changes inhibited in a mutant unable to germinate in L-proline (Rossignol and Vary, 1979; Skomurski *et al.*, 1983).

Mutants of *B. subtilis* that produce spores unable to respond to normal germination stimuli have been isolated. Spores of *B. subtilis* have two major germinant systems: either L-alanine or a mixture of glucose, fructose, asparagine and KCl (GFAK). *gerA* and *gerC* mutants germinate normally in GFAK but not in L-alanine; conversely, *gerB* and *gerK* mutants germinate normally in L-alanine and not in GFAK (Piggott *et al.*, 1981; Moir *et al.*, 1985). The *gerD*, *E*, *F*, *G*, *H*, *I*, *J* and *M* mutants comprise a heterogeneous group whose response to both the L-alanine and GFAK germinant systems is altered at a later stage of germination. These observations suggest that the L-alanine and GFAK pathways converge with *gerA*, *B*, *C* and *K* required for the initial trigger reactions and *gerD*, *E*, *F*, *G*, *H*, *I*, *J* and *M* involved later in the germination response. One method of establishing the direct functional involvement of the products of a *ger* locus in the germination pathway is to identify temperature-sensitive germination mutants. One such locus, *gerA*, has been cloned and sequenced (Zuberi *et al.*, 1987) and consists of three complementation units. On the basis of predicted amino acid sequences, it has been suggested that these three proteins may form a membrane-bound, multi-subunit receptor complex. In addition, the *gerC* locus has been cloned and is currently being sequenced (A. Moir, personal communication).

An alternative strategy towards identification of the receptor is to identify those events that occur as a result of its stimulation. A substantial body of evidence suggests that neither metabolism of the germinant nor germinant-stimulated metabolism are required for germination triggering in spores of *B. megaterium* and *B. subtilis*. Non-metabolizable germinant analogues, including PCK, are capable of initiating germination and no incorporation of label from radiolabelled germinants occurs during germination triggering (Rossignol and Vary, 1979; Scott and Ellar, 1978b). Mutant spores of *B. subtilis* deficient in L-alanine dehydrogenase, glycolytic enzymes and glucose dehydrogenase all germinate normally in both L-

alanine and GFAK (Prasad, 1974; Prasad *et al.*, 1972; Rather and Moran, 1988). Although it has been reported that phosphoglycerate kinase activity is necessary for L-alanine-induced germination of spores of *B. subtilis* (Prasad *et al.*, 1972), this has not been shown definitively by, for example, insertional activation of the structural gene. However, there is one well-characterized exception, *Bacillus fastidiosus*, which requires uricase activity for uric acid-induced germination (Salas *et al.*, 1985). No significant change in the pools of spore metabolites including ATP, NADH and TCA-cycle intermediates and no irreversible incorporation of tritium from tritiated water into spore metabolites occurs during triggering of *B. megaterium* spore germination (Scott and Ellar, 1978a,b). Also, a wide range of metabolic inhibitors, including KCN and KF, has no effect on germination of spores of *B. megaterium* and *B. subtilis* (Rossignol and Vary, 1979; Venkatasubramanian and Johnstone, 1989). These observations support the hypothesis that the germinant receptor is allosterically activated to initiate a cascade of hydrolytic reactions, resulting in germination.

Inter-relationship between germination events

Determination of the sequential inter-relationships between the early germination events is essential for an understanding of the trigger reaction in relation to the overall germination response. During germination, the spore concomitantly loses many of its characteristic properties including refractility, resistance and dormancy. A number of biochemical events accompany these changes which, during *B. megaterium* KM spore germination, can be divided into two categories: first, those early events, including commitment, loss of heat resistance and DPA release, which can be detected within one minute after addition of germinants and which may be associated, therefore, with the germination-triggering event; and secondly those events, including selective cortex hydrolysis, loss of soluble hexosamine, decrease in A_{600} , net ATP synthesis and the onset of general spore metabolism, which are initiated at a later time (Johnstone *et al.*, 1982; Foster, 1986). Thus the germination response comprises a series of interdependent biochemical events.

The relative asynchrony and heterogeneity of the germinating spore population makes precise sequencing of germination events impractical by direct analysis. The potent germination inhibitor mercuric chloride (HgCl_2) has been used to dissect the germination pathway further. Two Hg^{2+} -sensitive sites were observed during germination of spores of *B. megaterium* QMB1551 (Rossignol and Vary, 1977). In *B. megaterium* KM, the first of these sensitive sites (site I) is involved before commitment in the germination pathway and is deduced to be the L-alanine binding site since it can be protected from the effects of

Hg^{2+} by D-alanine (Foster and Johnstone, 1986). The second sensitive site (site II) is involved after commitment and probably results from inhibition of a cortex-lytic enzyme. In the presence of 1 mM HgCl_2 , 30% of the spore population becomes committed to germinate, whereas <5% of post-commitment events including DPA release, A_{600} loss and cortex hydrolysis occurs. Using these differential sensitivities of the two sites, loss of heat resistance by the spore population was shown to be the only commitment-associated event, possibly caused by the creation of a heat-sensitive active enzyme (Foster and Johnstone, 1986). Protease inhibitors arrest germination of *Bacillus cereus* at an early stage (Boschwitz *et al.*, 1985) and have been shown to inhibit *B. megaterium* KM spore germination at a pre-commitment site (Foster and Johnstone, 1986). Thus, proteolytic activity is implicated as a part of the triggering process.

The role of cortex-lytic enzymes

Other hydrolytic reactions remain strong candidates for involvement in germination triggering. Activation of a cortex-lytic enzyme has been suggested, by several workers, (e.g. Powell and Strange, 1956) to have a central role in the germination pathway. Cortex-lytic enzymes of different specificities have been isolated from spores of several species, but their involvement in germination has not been unequivocally established (Ando and Tsuzuki, 1984; Brown *et al.*, 1977). The germination-specific, cortex-lytic enzyme (GSLE) of *B. megaterium* KM purified from germinating spores has been strongly implicated as an essential component of the germination response (Foster and Johnstone, 1987). In the dormant spore, GSLE is present as an inactive 63 kD pro-form covalently bound to the spore-cortex peptidoglycan (Foster and Johnstone, 1988). During germination, active 30 kD enzyme is released which selectively hydrolyses the cortical peptidoglycan. This enzyme is most probably an amidase, since it causes selective cortex hydrolysis, as evidenced by its ability to cause an increase in the number of muramic acid δ -lactam residues in the spore cortex in the absence of germinants (Foster and Johnstone, 1987), which is a known early germination event (Johnstone and Ellar, 1982). GSLE has a very high substrate specificity and can only hydrolyse intact, *in situ*, spore-cortex peptidoglycan. Thus the spore-specific peptidoglycan muramic acid δ -lactam residues may play a role in GSLE binding-site recognition. A requirement for stressed peptidoglycan substrates has previously been suggested as a regulatory mechanism for autolysin activity (Koch, 1985). The inhibitor profile of GSLE activity and loss of absorbance during germination also show a high level of correlation. GSLE is Hg^{2+} -sensitive and thus probably represents the site II of Hg^{2+} inhibition during germination. Inhibitor studies have

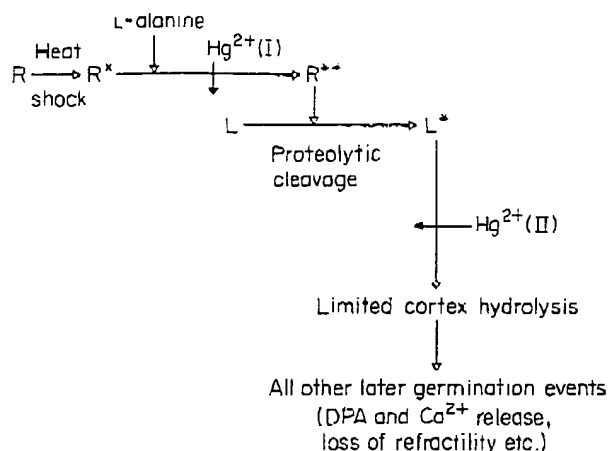


Fig. 2 Model for the germination pathway of *B. megaterium* KM (Foster and Johnstone, 1986), based on previous germination models (Stewart *et al.*, 1981; Johnstone *et al.*, 1982).

also shown the activation of GSLE to be associated with the commitment event, but sensitive to protease inhibitors. Thus current evidence suggests that proteolytic activation of GSLE forms part of the commitment reaction in spores of *B. megaterium* KM. Disruption of the structural GSLE gene will be necessary, however, to unequivocally demonstrate the proposed involvement of GSLE in the germination pathway. Anti-GSLE sera shows cross-reactivity with spore proteins of other organisms and therefore similar enzymes may be present in these species (Foster and Johnstone, 1988).

A model for germination triggering

The following model for the germination pathway of *B. megaterium* KM was proposed (Foster and Johnstone, 1986), based on previous germination models and the results described above (Stewart *et al.*, 1981; Johnstone *et al.*, 1982) (Fig. 2). This model has now been substantiated by further work (Foster and Johnstone, 1987; 1988).

Heat shock activates the L-alanine receptor ($R \rightarrow R^*$), which becomes triggered by L-alanine ($R^* \rightarrow R^{**}$). The L-alanine receptor has been identified as the first site of HgCl_2 inhibition (site I). R^{**} has proteolytic activity, which converts a pro-enzyme (L) to an active, heat-sensitive, cortex-lytic enzyme (L^*), which is also HgCl_2 -sensitive (site II). Commitment may represent the $L \rightarrow L^*$ reaction, and the loss of heat resistance prior to commitment is due to the heat sensitivity of R^{**} or L^* . Cortex hydrolysis then allows uptake of water and the onset of all other downstream germination events, including loss of Ca^{2+} and DPA, loss of spore refractility, release of soluble hexosamine and the onset of core metabolism. Very low

levels of cortex hydrolysis, which cannot be detected by existing techniques, may allow the release of small amounts of DPA from the spore core early in germination (Johnstone and Ellar, 1982). Thus the cortex-lytic enzyme regulates rehydration of the spore core during germination. Once water enters the spore core, it may solubilize and allow release of CaDPA as well as rehydrating core proteins and initiating the onset of general spore metabolism. More extensive cortex hydrolysis will allow the release of peptidoglycan fragments, which occurs as a late germination event (Dring and Gould, 1971; Johnstone *et al.*, 1982).

A common mechanism for spore germination?

During the past four decades, a substantial body of experimental data concerning the mechanism of germination of endospores of many species has been accumulated. A realistic starting point for inter-relating these data is to assume a common germination mechanism for spores of all species. Such a hypothesis is supported by several spore characteristics. Overall spore structure is very similar in all species (Fig. 1) and, in particular, the spore cortex peptidoglycan has a unique structure that is highly conserved. The osmoregulatory constraint imposed on the core by the spore cortex is considered to be the primary mechanism whereby spore dormancy is maintained and elimination of this constraint leads to the typical germination events. Also, the overall germination events are common to all species. Thus cortex hydrolysis may constitute a key event in spore germination of all species. Evolutionary divergence has led to the many different specificities of the germinant receptor and if cortex-lytic enzymes are activated during triggering, they may also show different specificities and modes of action; however, the underlying germination principle may be universal.

The model proposed above is the 'backbone' which forms the basis of the search for functional components involved in germination in organisms other than *B. megaterium* KM. Identification of the role of the gene products of the *gerA* operon, which codes for components essential for the L-alanine response in *B. subtilis* (Zuberi *et al.*, 1987), may now allow their relationship to as yet unidentified spore components responsible for the breaking of spore dormancy to be determined in this organism.

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The trigger mechanism of spore germination: current concepts

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1. INTRODUCTION

The metabolic dormancy and the resistance properties of bacterial spores are both crucial to the ecological role of spores as survival structures. In order to complete this role it is also essential that spores are able to monitor their external environment so as to trigger germination in suitable environmental conditions. Thus paradoxically, in the absence of metabolic processes, the spore must retain an alert sensory mechanism which is able to initiate the germination process (Gould 1983). Metabolic dormancy and heat resistance are imposed on the spore core by a number of mechanisms (Gerhardt and Marquis 1989). These include immobilization of core macromolecules, enzymes and metabolites in a dehydrated calcium dipicolinate gel (Stewart *et al.* 1979; Stewart *et al.* 1980; Johnstone *et al.* 1980, 1982a). In addition the inner spore membrane is present in a semi-crystalline state (Stewart *et al.* 1979). The germination sensing mechanism must be able to function in the absence of general metabolic processes and thus must escape the mechanisms of dormancy and resistance generally imposed on spore constituents. This mechanism may therefore be located outside the spore core and inner membranes and yet must intrinsically possess the resistance properties of the intact spore.

This article reviews our current knowledge of the spore germinant sensing mechanism by building a conceptual framework from selected research articles to produce a working model of this mechanism. It does not attempt to review the vast wealth of spore germination literature—the reader is referred to reviews concerning spore structure

(Ellar 1978; Keynan 1978; Warth 1978; Russell 1982), the genetics of germination (Moir *et al.*, this Symposium, pp. 9S-16S; Smith *et al.* 1977; Moir and Smith 1985, 1990) and the biochemistry of germination (Foster and Johnstone 1989b, 1990) for this purpose.

2. GERMINATION AND THE GERMINANT RECEPTOR

In this article the definition of germination as 'a series of degradative events triggered by specific germinants which leads to the loss of typical spore properties' proposed by Foster and Johnstone (1990) will be adopted. Germination can be induced by a variety of processes including exposure to nutrient germinants such as amino acids and sugars, to non-nutrient germinants including dodecylamine, to enzymes and to hydrostatic pressure (Gould 1969). This article will focus on the nutrient germinants, the biochemistry of which has been most intensively studied and which represent the physiological germination pathway. The nutrient germinants range from the simple amino acid L-alanine commonly required for triggering of germination of *Bacillus* species (Harrell and Halvorson 1955) to complex mixtures including amino acids, sugars and ions typical of *Clostridium* spp. (Bright and Johnstone 1987).

The stereospecific properties of the germinant receptors demonstrate that this component of the trigger mechanism must be a protein, which may be activated allosterically (Wolgamott and Durham 1971). Typically the germinant receptors are 50% saturated by 50-100 $\mu\text{mol l}^{-1}$ concentrations of germinants (Bright and Johnstone 1987; Venkatasubramanian and Johnstone 1989). Other properties of the germinant receptor have been relatively poorly studied.

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strates that the spore population rapidly undergoes the commitment reaction but that the time window between commitment and germination is much more variable. Identification of the mechanism of germination triggering is thus concerned with analysis of the events which take place within the first minutes of exposure to specific germinants.

4. BIOCHEMICAL CHARACTERIZATION OF THE COMMITMENT REACTION

Several approaches have been employed to identify the biochemical events which occur during germination triggering. The knowledge gained from three successful approaches is summarized below.

4.1 Germinant metabolism

The question as to whether metabolism of germinants is required for germination triggering has generated much controversy. Three strategies have been employed to address this question. First, a range of non-metabolizable germinant analogues has been shown to induce germination in spores of several organisms. These include L-proline chloromethyl ketone in place of L-proline in *B. megaterium* QMB1551 (Rossignol and Vary 1978, 1979), L-alanine chloromethyl ketone as a substitute for L-alanine in *B. megaterium* KM (Foster 1986), 2-deoxy-D-glucose and D-allose as a substitute for glucose in *B. subtilis* (Prasad *et al.* 1972) and allylglycine and cycloleucine as a substitute for L-alanine in *B. subtilis* (Woese *et al.* 1958; Irie *et al.* 1980, 1982; Sammons *et al.* 1981; Yasuda and Tochikubo 1985; Kanda *et al.* 1988). Second, the ability of mutants deficient in enzymes in germinant metabolic pathways to germinate has been studied. Mutants of *B. subtilis* lacking L-alanine dehydrogenase (Freese and Cashei 1965), pyruvate dehydrogenase (Freese and Fortnagel 1969) or glucose dehydrogenase (Rather and Moran 1988) all germinate normally. The third approach has been to analyse metabolism of the germinants themselves. However no incorporation of radioactivity into other compounds from labelled L-alanine and from D-glucose in *B. megaterium* KM (Scott and Ellar 1978b) and *B. megaterium* QMB1551 (Shay and Vary 1978) spore germination respectively was detected. It may therefore be concluded that metabolism of the germinants is not required for germination triggering. There is however one documented exception to this general rule: in spores of *B. fastidiosus*, uricase has been shown to be required for uric acid induced germination (Salas *et al.* 1985).

4.2 Identification of metabolic events during germination triggering

An alternative approach which has been used to examine events associated with the germination triggering and com-

mitment reactions in spores of *B. megaterium* KM, is to look for biochemical and physiological changes during the first minutes of germination. These changes can be conveniently divided into two categories (Foster and Johnstone 1989b): (1) those events including loss of heat resistance, commitment and dipicolinic acid (DPA) release which are detected within the first minute of germination triggering and which may therefore be associated with the trigger reaction; and (2) those events including O.D.₆₀₀ loss, selective cortex hydrolysis and the onset of spore metabolism which are initiated at a later time and are presumably not therefore directly associated with triggering.

No significant changes in spore metabolite pools including tricarboxylic acid intermediates, ATP, NADH and NADPH, were identified during triggering of *B. megaterium* KM spore germination (Scott and Ellar 1978a). Furthermore no detectable irreversible incorporation of protons from tritiated water into spore metabolites was observed during the first minutes of germination (Scott and Ellar 1978b; Johnstone *et al.* 1982b). Thus activity of the major metabolic pathways is not required for germination triggering. This view is supported by the lack of effect of a wide range of metabolic inhibitors on germination (see below).

4.3 Inhibitor studies

Metabolic inhibitors are potentially powerful tools to identify essential metabolic events during germination triggering. For this purpose it is necessary to establish that a metabolic inhibitor blocks the commitment reaction. This has been achieved in studies of *B. megaterium* KM spore germination (see below). In several species, germination has been shown to occur in the presence of a wide range of metabolic inhibitors including inhibitors of DNA, RNA and protein synthesis, of glycolysis and of the respiratory chain (Dills and Vary 1978; Scott *et al.* 1978; Rossignol and Vary 1979). The results of such metabolic inhibitor experiments must however be interpreted with caution since the lack of effect of an inhibitor may be due to its inability to access the target site. In contrast, HgCl₂ is a potent reversible inhibitor of germination in several species including *B. megaterium* (Levinson and Hyatt 1966; Foster and Johnstone 1986). Evidence has also been presented that protease inhibitors block early steps of germination in spores of *B. cereus* T (Boschwitz *et al.* 1983, 1985) and germination triggering in spores of *B. megaterium* KM (Foster and Johnstone 1986). These findings therefore suggest that a component of the germination trigger contains essential sulphhydryl groups and may have a proteolytic activity.

The HgCl₂ sensitivity of *B. megaterium* KM spore germination has been extensively characterized. Two HgCl₂ sensitive sites are present in the germination pathway

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(Foster and Johnstone 1986). The first (site I) is a pre-commitment event and can be protected from HgCl_2 by 50 mmol l^{-1} D-alanine, which suggests that it is part of the trigger reaction. The second (site II) is a post-commitment event and cannot be protected by D-alanine. Due to the differential sensitivity of these two sites it was demonstrated that in the presence of 1 mmol l^{-1} HgCl_2 , 25% of the spore population becomes committed to germinate whereas DPA, Ca^{2+} , Zn^{2+} and peptidoglycan release as well as loss of refractility and selective cortex hydrolysis are >95% inhibited. This commitment reaction, which occurs in the presence of 1 mmol l^{-1} HgCl_2 was, however, inhibited by the presence of protease inhibitors. Thus in *B. megaterium* KM, the commitment reaction can be identified as one which occurs in the presence of 1 mmol l^{-1} HgCl_2 , but which is protease inhibitor sensitive.

5. PEPTIDOGLYCAN HYDROLYSIS DURING GERMINATION TRIGGERING

Given the central role of the spore cortex in maintaining spore dormancy by maintaining the dehydrated state of the spore core (Ellar 1978; Warth 1978), hydrolysis of the spore cortex peptidoglycan might be expected to occur early during germination. Activation of a cortex lytic enzyme as a primary event in spore germination was originally suggested by Powell and Strange (1956). This view is supported by the observation that germination can be initiated by peptidoglycan lytic enzymes if the spore coats are naturally permeable to the enzyme (Suzuki and Rode 1969) or are rendered permeable by chemical treatment (Gould and Dring 1972). The spore cortex peptidoglycan is structurally distinct from that of the vegetative cell and in particular it is less extensively cross-linked (Rogers 1977). Its structural integrity is therefore likely to be significantly altered by selective hydrolysis of either the glycan chains or the peptide cross-links as a result of glycosylase or peptidase activities respectively. Although HgCl_2 prevents release of soluble peptidoglycan fragments during germination (Rossignol and Vary 1978), cortex hydrolysis has been considered to be a late event during germination (Dring and Gould 1971; Hsieh and Vary 1975). Such analysis was based on the release of soluble peptidoglycan fragments and would be unlikely to detect selected limited hydrolysis of the spore cortex which could occur early in germination. Measurement of reducing termini during germination of spores of *B. megaterium* KM showed that selective cortex hydrolysis could be detected within 2 min of addition of germinants (Johnstone and Ellar 1982), providing evidence that cortex hydrolysis might constitute an early germination event.

Two classes of cortex lytic enzymes have been purified from dormant and germinating spores. First, surface bound

enzymes which are able to hydrolyse isolated spore cortex but are unable to induce germination-like changes in permeabilized spores (Srivastava and Fitz-James 1981; Brown *et al.* 1982; Foster and Johnstone 1987). Since spores germinate normally after extraction of such enzymes, these surface bound enzymes appear to play no direct role in the germination process. Secondly, enzymes which are able to induce germination in permeabilized spores have been extracted from both germinating and broken spores (Gombas and Labbe 1981; Brown *et al.* 1982; Ando and Tsuzuki 1984). The best characterized of this second class of enzymes is the germination-specific cortex-lytic enzyme (GSLE) of *Bacillus megaterium* KM (Foster and Johnstone 1987). There is substantial biochemical evidence that GSLE plays a key role in germination and that proteolytic activation of GSLE from a 68-kDa cortex-associated pro-form to yield a 29-kDa active enzyme constitutes part of the germination trigger mechanism (Foster and Johnstone 1989a, 1989b). This includes:

- (1) Substrate specificity: GSLE is only active on the cortex of intact spores. No activity is observed on isolated spore peptidoglycan or on vegetative cell walls. GSLE thus has a specific requirement for intact stressed cortex peptidoglycan as a substrate.
- (2) Mechanism of action: incubation of GSLE with permeabilized spores in the absence of germinants results in an increase in spore cortex muramic acid δ -lactam reducing termini as is observed during initiation of germination.
- (3) Inhibitor profile: purified GSLE shows the same Hg^{2+} inhibition characteristics as the post-commitment site II HgCl_2 sensitive site.
- (4) Activation during germination triggering: activation of GSLE occurs in the presence of 1 mmol l^{-1} HgCl_2 and its activation is inhibited by 1 mmol l^{-1} PMSF. This inhibition profile parallels that of the commitment reaction as described above.

Activation of GSLE therefore conforms to the biochemical criteria for it to be considered part of the germination triggering mechanism.

6. A MODEL FOR GERMINATION TRIGGERING

A model for the germination triggering reaction in spores of *B. megaterium* KM which is based on models described previously (Stewart *et al.* 1981; Foster and Johnstone 1989b, 1990) and which combines the key findings described above, is shown in Fig. 3. In this model the germination receptor (R) is altered conformationally by heat shock such that it is more responsive to the presence of the

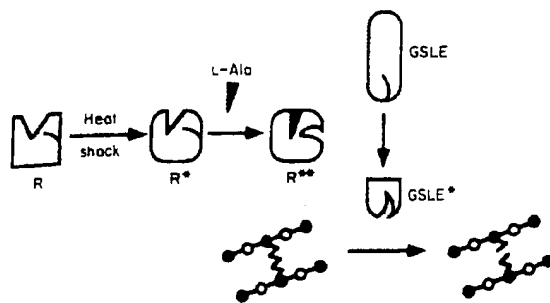


Fig. 3 Model for germination triggering in spores of *Bacillus megaterium* KM. For details see text. Based on previous models described by Stewart *et al.* (1981) and Foster and Johnstone (1989a, b)

germinant L-alanine. The rates of both commitment and germination have been shown to be stimulated by heat shock in this organism (Stewart *et al.* 1981). Binding of L-alanine induces a further conformational change in the germinant receptor which activates its PMSF-sensitive proteolytic site (R**). It is deduced that the activity of the protease is Hg^{2+} -sensitive and constitutes site I of Hg^{2+} inhibition described above. The active protease cleaves the 68-kDa pro-form of peptidoglycan-immobilized GSLE to release the active heat sensitive 29-kDa enzyme. Depolymerization of the spore cortex is catalysed by GSLE, which is also Hg^{2+} sensitive and represents site II of Hg^{2+} inhibition described above. Cortex depolymerization results in uptake of water into the spore core, the release of spore ions including DPA and Ca^{2+} , and the onset of spore metabolism. In this model, germination triggering requires no metabolism of the germinant L-alanine and takes place in the absence of general spore metabolism; it is driven by a series of exergonic hydrolytic reactions. The first irreversible event in the pathway is the activation of GSLE; commitment thus represents the proteolysis of sufficient GSLE such that a spore will germinate within the time window defined for commitment. Once GSLE has been activated, the subsequent germination pathway is an inevitable process. Since GSLE is Hg^{2+} -sensitive, it probably corresponds to the post-commitment Hg^{2+} -sensitive site described above. It is not known whether there are additional steps between R** and GSLE*; for example a proteolytic cascade might be involved.

Although this germination pathway has been extensively characterized in spores of *B. megaterium* KM, the question arises as to whether it is common to spores of other organisms. The general principle of germinant-mediated proteolytic activation of a peptidoglycan hydrolytic enzyme to initiate the germination response is supported by a

number of observations in spores of other organisms. These include the conserved structure of spore cortex peptidoglycan, the identification of pre- and post- Hg^{2+} -sensitive sites in other germination pathways (Venkatasubramanian and Johnstone 1989), the observed release of peptidoglycan fragments during germination and identification of a commitment reaction. There is, however, a growing body of evidence that the mechanism of cortex hydrolysis during germination of spores of other organisms differs from that observed in *B. megaterium* KM. An increase in cortex muramic acid δ -lactam content was not found during germination of spores of *B. subtilis* (Venkatasubramanian and Johnstone 1989), *B. fastidiosus* (Salas *et al.* 1985), *B. megaterium* ATCC 12872 (Nakatani *et al.* 1985) and *Clostridium bifermentans* (Bright and Johnstone, unpublished). In the case of *B. megaterium* ATCC 12872, an increase in cortical glucosaminol content was observed during germination, which suggests activation of a glucosamidase during triggering; in the other spores no change in the reducing termini of residual spore peptidoglycan was detected. It is therefore likely that cortex depolymerization occurs by endopeptidase or transpeptidase activity in these organisms.

7. CONCLUSIONS

During the past decade the signal transduction pathways whereby micro-organisms detect and respond to environmental stimuli have been extensively studied and a number of response mechanisms have been identified (Parkinson 1993). These include protein phosphorylation via conserved two-component sensors and regulators which induce changes in protein function at the level of transcription (e.g. osmoregulation) or by directly influencing protein function (e.g. the chemotactic response). Alternatively, substitution of sigma factors may alter patterns of gene expression (e.g. during sporulation; Kaiser and Losick 1993). Although allosterically activated proteolytic cleavage is well established as a mechanism of intracellular signalling in bacteria (e.g. the *lonA* activity in the SOS response; Little and Mount 1982), a germination triggering mechanism based on allosterically-induced proteolytic cleavage represents a novel prokaryotic environmental sensing mechanism.

There are several key questions which remain to be answered concerning the spore germination trigger reaction. These include identification of the location of the receptor in the spore and demonstration *in vitro* of germinant-dependent catalytic activity of the germinant receptor. In addition, in order to establish the validity of the model proposed above it will also be necessary to examine the effects on germination of mutations in the

GSLE structural gene. Experiments are currently in progress to these ends.

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Resistance, Germination, and Permeability Correlates of *Bacillus megaterium* Spores Successively Divested of Integument Layers†

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A variant strain that produced spores lacking exosporium was isolated from a culture of *Bacillus megaterium* QM-B1551. Two additional spore morphotypes were obtained from the parent and variant strains by chemical removal of the complex of coat and outer membrane. Among the four morphotype spores, heat resistance did not correlate with total water content, wet density, refractive index, or dipicolinate or cation content, but did correlate with the volume ratio of protoplast to protoplast plus cortex. The divestment of integument layers exterior to the cortex had little influence on heat resistance. Moreover, the divestment did not change the response of either the parent or the variant spores to various germination-initiating agents, except for making the spores susceptible to germination by lysozyme. The primary permeability barrier to glucose for the intact parent and variant spores was found to be the outer membrane, whereas the barrier for the divested spores was the inner membrane.

Investigation of the mechanisms by which bacterial spores achieve resistance and initiate germination has been complicated by the use of model types of spores that possess superfluous integument layers, such as spores of *Bacillus cereus* T, *Bacillus subtilis* 168, and *Bacillus megaterium* QM-B1551. Consequently, it appeared desirable to obtain structurally simplified dormant spores derived from a single, well-studied strain to serve as models for further investigation.

In this paper, we report the isolation from *B. megaterium* QM-B1551 of a variant strain that produces spores devoid of the thick peripheral structure which we term exosporium. Both the parent and the variant spores were chemically divested of the coat and outer membrane, the variant spore thus becoming a cortex-encased protoplast. The four morphotype spores, all dormant and heat resistant, were examined for fine structure by electron microscopy, studied for biophysical and biochemical correlates of heat resistance, tested with chemical agents that initiate germination, and used to distinguish the permeability roles of the outer and inner membranes.

MATERIALS AND METHODS

Organisms. *B. megaterium* QM-B1551 (ATCC 12872) was obtained from James C. Vary, University of Illinois Medical Center, Chicago. From this parent strain, an apparently naturally occurring variant that produced spores lacking exosporium (EX⁻ variant) was isolated by chance selection during serial transfer. The genetic basis was not studied. However, tests conducted at the American Type Culture Collection, Rockville, Md., indicated that the variant has identifying characteristics like those of authentic *B. megaterium* QM-B1551 (Robert L. Gherna, personal communi-

cation). The variant is available from the Collection under accession number 33729.

Spore production. Dormant spores of the parent and variant *B. megaterium* strains were produced essentially by the procedure of Shay and Vary (34). Vegetative cells grown overnight on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) were inoculated into 300 ml of supplemented nutrient broth (33) and incubated with aeration by shaking for 7 h at 30°C. The culture was then transferred into 10 liters of the same medium in a fermentor and incubated for 24 h at 30°C with constant stirring (400 rpm) and aeration (11 liters of air per min). The resulting spores were harvested by centrifugation (5,000 × g for 5 min) and washed by centrifugation about 15 times with cold, sterile, deionized water; each time, the supernatant liquid was decanted and debris was removed from the surface of the pellet. The spores were stored in water at 4°C and washed twice daily by centrifugation. The four morphotype spores all remained viable, refractile, and heat resistant when stored in this way.

Electron microscopy. Specimens were prepared, stained, sectioned, and examined by electron microscopy essentially as described previously (4).

Volume fractions. Estimates of the volume percentage of the spore occupied by the protoplast and the sporoplast (essentially, the protoplast plus cortex; actually, the protoplast plus primordial cell wall plus cortex plus coat underlayer) were obtained by calculation from measurements on electron micrographs, care being taken to select only longitudinal center sections of spores, as described previously (3).

Chemical treatment. Spores of the parent and the variant were both chemically treated to remove the complex of coat and outer membrane (C⁻ OM⁻), essentially as described by Fitz-James (14) and Vary (39). Clean spores (10 g, wet weight) were suspended in a freshly made solution of 0.5% sodium dodecyl sulfate–0.1 M dithiothreitol–0.1 M NaOH (pH 10). The suspension was shaken (150 rpm) for 2 h at 37°C, and the spores were harvested by centrifugation (5,000

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$\times g$ for 10 min). The resulting spores were then washed and stored as described above.

Determination of heat resistance. Heat resistance of each of the four morphotype spores was determined at 70, 80, 90, and 100°C and expressed as a D value, as described previously (3).

Water properties. Determinations of wet density and water content by direct gravimetric and volumetric measurements were made as described previously (3), except for the initial centrifugation conditions. The parent spores were centrifuged for 30 min at $11,000 \times g$, and the variant spores were centrifuged for 30 min at $4,400 \times g$.

Refractometry. Determinations of average apparent refractive index were obtained by photometric immersion refractometry as described previously (19). Changes in optical density (OD) were monitored by means of a double-beam spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) operated at a wavelength of 700 nm with deionized water as a blank.

Chemical analyses. Dipicolinic acid was determined by the method of Janssen et al. (25). Hexosamine was determined by the method of Cessi and Piliego (11) after hydrolysis of the spores in 3 N HCl for 4 h at 95°C. Mineral element analyses (Ca^{2+} , Mg^{2+} , Mn^{2+} , K^+ , Na^+) were made by use of atomic absorption spectroscopy (27a).

Initiation of germination. Initiation of germination was studied with various chemical agents and determined by measuring the decrease in OD, essentially as described by Vary (39), so as to be comparable. A concentrated suspension of spores (OD, 6.0) was heat activated for 10 min at 60°C, chilled in an ice bath, and diluted with initiation medium contained in a cuvette to a final OD of 0.6, which was within the proportionality range of the spectrophotometer. Final reagent concentrations were as follows: 5 mM Tris buffer; 16 mM phosphate buffer; 10 mM each D-glucose, L-leucine, L-proline, and KNO_3 ; 2 mM inosine; 200 μg of lysozyme per ml. The decrease in OD was measured at 660 nm, with readings taken initially and after 30 min of incubation at 30°C. The results were expressed as the percentage of decrease in OD and were confirmed by observation of the loss in spore refractility by use of a phase-contrast microscope. An OD decrease of greater than 45% was associated with greater than 90% conversion to phase-dark spores, and an OD decrease of less than 10% was associated with less than 10% conversion to phase-dark spores. The determinations were replicated two to four times, and average values were reported.

Permeability. Permeability measurements were made with ^3H -labeled water and ^{14}C -labeled D-glucose, with correction for the amount of interstitial water obtained from a similar measurement with dextran of high molecular weight (M_n , 2,000,000), as described previously (19). Equilibrium uptake of the labeled water and glucose was attained within 1 h and maintained for at least 24 h by all of the morphotype spores (data not shown). The suspension of spores and solution was routinely allowed to equilibrate for 2 h at 4°C. The native parent and the C⁻OM⁻ parent spores were then centrifuged at $11,000 \times g$ for 30 min, whereas the EX⁻ variant and the EX⁻C⁻OM⁻ variant spores were centrifuged at $4,400 \times g$ for 30 min. In both cases, the supernatant solution was clarified by centrifugation at $33,000 \times g$ for 30 min. Radioactivity was measured with samples of the supernatant solution appropriately diluted in aqueous scintillation fluid (New England Nuclear Corp., Boston, Mass.) and counted in a scintillation counter (Beckman Instruments). The results were expressed as the volume percentage of the spore

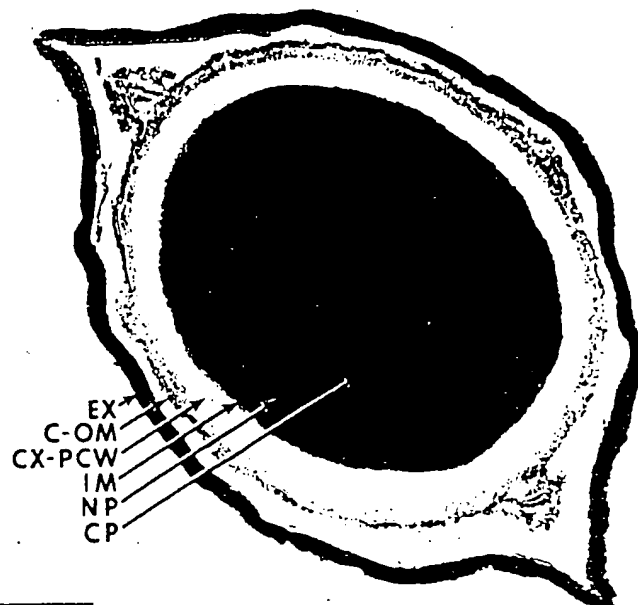


FIG. 1. Stained sectioned parent spore of *B. megaterium* QM-B1551 shown by transmission electron microscopy. This morphotype spore contained all of the usual structural components: exosporium (EX), complex of coat and outer membrane (C-OM), complex of cortex and primordial cell wall (CX-PCW), inner membrane (IM), DNA-containing nucleoplasm (NP), and ribosome-containing cytoplasm (CP). The protoplast consists of the cytoplasm and nucleoplasm enclosed by the inner membrane; the integument consists of everything outside of the protoplast. Bar, 200 nm.

permeated by the labeled water or glucose on a wet-weight basis (R^w ; expressed as milliliters per 100 g of wet spore = grams per 100 g of wet spore) or on a wet-volume basis (R^v ; expressed as milliliters per 100 ml of wet spore). R^w and R^v were interconvertible by use of the appropriate wet-density value. The R^w was equivalent to the water content on a wet-weight basis, which was directly comparable to that obtained by the gravimetric method. The R^v for glucose was converted to a glucose-impermeable volume percentage ($100 - R_{\text{glucose}}$) for comparison with the physical volume percentage calculated from the measurements made on electron micrographs.

RESULTS

Fine structure. The parent strain of *B. megaterium* QM-B1551 produced spores that contained all of the usual components in stained and sectioned fine structure (Fig. 1) and were consistent in appearance with prior electron micrographs of this strain (4, 17) and morphologically similar strains (2, 15). The outstanding feature was a thick, loose-fitting, peripheral structure with apical openings. This structure often is termed outer coat but should be termed exosporium (see Discussion). The atypical exosporium of this spore lacked the thick, hairlike nap outside of the basal layer, which is present in the typical exosporium of spores of *B. megaterium* Mg19 (4) and of *B. cereus*, *B. anthracis*, and *B. thuringiensis* (18, 20, 21, 29).

Electron microscopy of the unstained intact parent spore of *B. megaterium* QM-B1551 revealed that the exosporium in itself was electron translucent (Fig. 2A).

After treatment of the parent spore with alkaline sodium dodecyl sulfate and dithiothreitol to remove the complex of

Fig. 7, together with previous results for five other types of spores (3). An exponential increase in heat resistance correlated with a decrease in the protoplast/protoplast-plus-cortex ratio over almost five decades of D_{100} values for the seven types of spores.

Concentrations of minerals and dipicolinic acid were determined in the native parent and EX⁻ C⁻ OM⁻ variant spores (Table 2). Specific mineralization is a major determinant of heat resistance in spores (27a). Dipicolinate occurs in high concentrations, but its function remains uncertain. Both morphotype spores contained about 0.5 μ mol of calcium per mg, and the ratio of calcium to dipicolinate was about 0.5. The magnesium content in the parent spore was higher than that in the variant spore, whereas the reverse was true for potassium. The two morphotype spores contained a total of 1.12 and 1.15 μ eq of cations per mg (dry weight) of spores, respectively. Both morphotype spores contained similarly high amounts of dipicolinate (about 1 μ mol/mg), equivalent to about 16% of the spore dry weight. A correlation of these constituents with heat resistance was not evident.

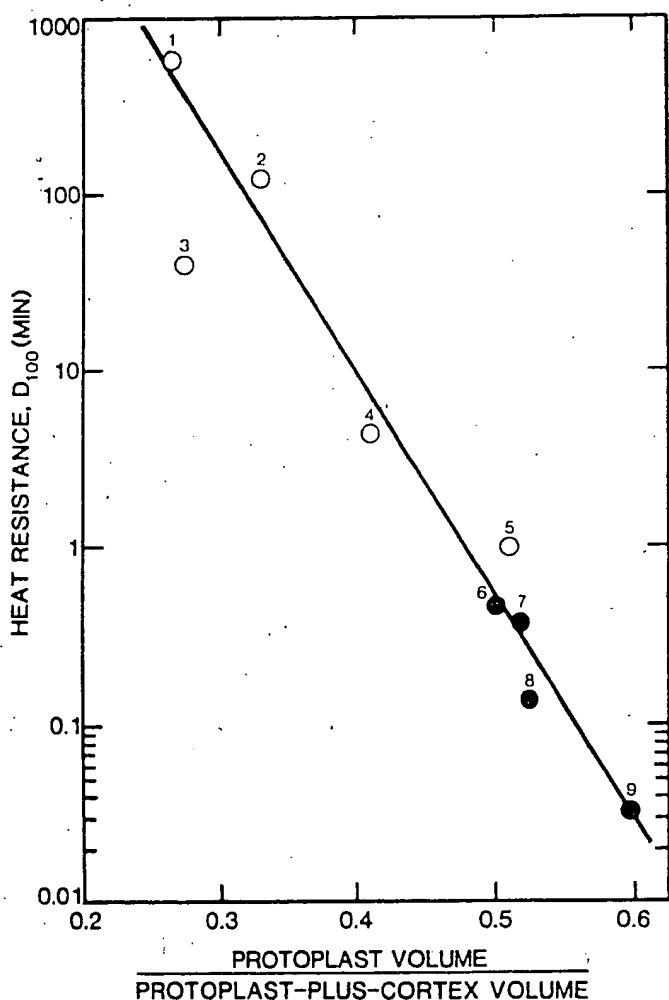


FIG. 7. Heat resistance of *B. megaterium* morphotype spores (●) and various other types of spores (○) (3) correlated by least-squares analysis with volume ratio of protoplast to protoplast plus cortex. The numbers correspond to the following types of spores: 1. *B. stearothermophilus* smooth; 2. *B. stearothermophilus* rough; 3. *B. subtilis* niger; 4. *B. cereus* T. high calcium; 5. *B. cereus* T. low calcium; 6. *B. megaterium* native parent; 7. *B. megaterium* C⁻ OM⁻ parent; 8. *B. megaterium* EX⁻ variant; 9. *B. megaterium* EX⁻ C⁻ OM⁻ variant.

TABLE 2. Mineral and dipicolinic acid contents of morphotype spores

Ion	Content (μ mol/mg of spore [dry wt]) in:	
	Native parent	EX variant
K	0.18	0.34
Na	0.03	0.08
Ca	0.48	0.54
Mg	0.41	0.17
Mn	0.01	0.02
Dipicolinate ^a	0.91	1.01

^a In the EX⁻ C⁻ OM⁻ variant, the dipicolinate content was 1.07 μ mol/mg of spore (dry weight).

Dipicolinate content was also determined in the EX⁻ C⁻ OM⁻ variant spore (1.07 μ mol/mg of spore [dry weight]). Because of the reduced dry-weight basis, this value was greater than that in the other two morphotype spores. Little dipicolinate was lost by chemical removal of the coat and outer membrane.

Germination. The four morphotype spores were examined for their responses to various germination-initiating agents after heat activation. The responses were more pronounced in phosphate (Table 3) than in Tris buffer (data not shown), but the patterns were similar. The native parent spore responded to glucose, leucine, proline, and glucose plus KNO₃, as was expected (31, 38). The EX⁻ variant spore, like several naturally occurring spores that lack exosporium (31), did not respond to these agents, but unlike the similar natural morphotype spores (31), also did not respond to alanine and inosine. This lack of response by the EX⁻ variant spore was not changed by chemical removal of the coat and outer membrane or by the addition of exosporium isolated from the C⁻ OM⁻ parent spore. Both the EX⁻ and EX⁻ C⁻ OM⁻ variant spores initiated germination (and grew out) in the complex of agents that are present in supplemented nutrient broth; however, 21 amino acids together, alone, or in various combinations were found to be ineffective (data not shown). Thus, the chemical germination requirements of the variant spore remain undefined.

The four morphotype spores were also examined for their response to lysozyme (Table 3), which can initiate germination only if it has access to the cortex. The enzyme then digests the cortex peptidoglycan, releasing its physical containment of the protoplast and causing the avalanche of

TABLE 3. Response of morphotype spores to germination-initiating agents in phosphate buffer

Agent	% Decrease in OD after 30 min in:			
	Parent		Variant	
	Native	C ⁻ OM ⁻	EX ⁻	EX ⁻ C ⁻ OM ⁻
None	11	4	12	5
D-Glucose	55	58	5	3
L-Leucine	46	18	4	3
L-Proline	52	20	4	2
D-Glucose + KNO ₃	56	68	4	2
D-Glucose + L-leucine	56	68	4	3
L-Alanine	10	6	5	2
Inosine	13	5	5	5
L-Alanine + inosine	17	13	3	4
Sporulation nutrient broth	55	70	33	37
Lysozyme	3	41	18	42

events associated with germination. The native parent spore did not respond to lysozyme, as was expected; the $C^- OM^-$ parent spore did so, however, because lysozyme had access to the cortex after penetrating the exosporium through apical openings. Likewise, the EX^- variant spore did not respond to lysozyme, but the $EX^- C^- OM^-$ variant spore did so because lysozyme had direct access to the cortex. Consequently, it was evident that the complex of coat and outer membrane functioned to prevent the penetration of lysozyme into the cortex of the intact parent and variant spores. In the complex, the peripheral layer of the coat apparently functioned as the primary permeability barrier to lysozyme, on the basis of previous observations on macromolecular sieving by spores (22).

Permeability. The morphotype spores were also used to distinguish the roles of the outer and inner membranes in spore permeability to a model small molecule. Equilibrium permeability measurements were made with ^{14}C -labeled D-glucose, on the basis of both wet-spore weight (R_{glucose}^w) and wet-spore volume (R_{glucose}^v), and similarly with 3H -labeled water (Table 4). Glucose might be thought to be unsuitable because of possible degradation by metabolism or uptake as a germinating agent; dormant spores are metabolically inactive, however, and germination did not occur under the restrictive conditions used in the permeability measurements. Furthermore, ^{14}C -labeled D-ribose and 3H -labeled 2-deoxy-D-glucose, neither of which initiates germination or is metabolized after germination by another agent, gave the same results.

In the native parent spore, glucose penetrated 43% of the spore volume (Table 4), an observation consistent with penetration of the large amount of peripheral integument (exosporium and coat) on this morphotype (see Fig. 1). In the EX^- variant spore, glucose penetrated only 11% of the spore volume, an observation consistent with penetration of the small amount of peripheral coat layer on this morphotype (see Fig. 4). Thus it was the outer pericortex membrane, not the inner pericytoplasm membrane, that apparently functioned as the primary permeability barrier to glucose in these morphotype spores.

Indeed, when the outer membrane and coat were chemically divested from the parent and variant spores, glucose penetrated to a much greater extent of the spore volume (53% in the $C^- OM^-$ parent and 28% in the $EX^- C^- OM^-$ variant). In these morphotype spores, glucose apparently penetrated through the cortex to the inner membrane, which now served as the primary permeability barrier.

An effort was made to quantify these comparisons of permeability function with spore structure. The respective R_{glucose}^v was converted to the glucose-impermeable percentage volume ($100 - R_{\text{glucose}}^v$) for comparison with the physical percentage volumes occupied by the protoplast and by the protoplast plus cortex as determined from electron microscopy measurements. The glucose-impermeable volume corresponded roughly with the physically measured volume of

the protoplast plus cortex in the native parent and EX^- variant spores and with the volume of the protoplast in the $C^- OM^-$ parent and $EX^- C^- OM^-$ variant spores (Table 5). The glucose-impermeable volume was only roughly comparable with the physically measured volume, partly because of the inaccuracy in calculating volumes from measurements on electron micrographs, but mainly because the R_{glucose}^v reflects only the volume occupied by water within a compartment, whereas the physically measured volume reflects the volume occupied by solids as well as water. Consequently, the R_{glucose}^v underestimates and the glucose-impermeable value overestimates the corresponding physically measured value. For example, with the EX^- variant, the glucose-impermeable value, corrected so as to take into account the volume occupied by solids as well as water, was 81%, which corresponded much better with the physically measured volume of 72% for the sporoplast than with that of 38% for the protoplast. For the $EX^- C^- OM^-$ variant, the corrected value was 56%, which corresponded much better with the physically measured volume of 60% for the protoplast than with that of 100% for the sporoplast.

Altogether, the results indicate that the outer pericortex membrane functioned as the primary permeability barrier to glucose in the native parent and EX^- variant spores, whereas the inner pericytoplasm membrane functioned as the permeability barrier to glucose in the $C^- OM^-$ parent and $EX^- C^- OM^-$ variant spores. The latter situation also occurs in two lysozyme-susceptible strains of *B. megaterium* spores in which the complex of coat and outer membrane is defective (T. C. Beaman, T. Koshikawa, H. S. Pankratz, and P. Gerhardt, FEMS Microbiol. Lett., in press). Furthermore, the results indicate that the exosporium in the parent spore and the coat peripheral to the outer membrane in the native parent or EX^- variant spores did not function as a permeability barrier to glucose.

The permeability measurements also provided an alternative method to the gravimetric method for determining water content, in that the R^w obtained with 3H -labeled water is equivalent to the total water content of the spore on a wet-weight basis (8; Beaman et al., in press). The values of 58, 56, 36, and 40% obtained by the permeability method (Table 4) were essentially the same as the values of 50, 58, 32, and 38% obtained by the gravimetric method for total water contents of the four morphotype spores, respectively (Table 1). The greatest discrepancy between the two methods (58% versus 50%) occurred with the native parent spore, which was encumbered with the greatest amount of integument layers (Fig. 1).

DISCUSSION

Heat resistance. The divestment of integument layers exterior to the cortex had relatively little influence on heat resistance among the four morphotype spores. In comparison with the D_{47} value of the germinated spore, the extrapolated value of the dormant spore was changed by only about 0.001% by removal of the exosporium, coat, and outer membrane (Table 1). Such a cortex-encased protoplast retains heat resistance also in coatless mutant spores (10) and divested spores (27) of *Clostridium* species. However, neither others nor we have accomplished the isolation of free spore protoplasts devoid of cortex that retain dormancy and heat resistance, despite various efforts; indeed, this may be intrinsically impossible.

The relationship between heat resistance and water con-

TABLE 4. Permeability^a of morphotype spores to [^{14}C]glucose and [3H]water

Morphotype spore	R^w		R^v	
	Glucose	Water	Glucose	Water
Native parent	35	58	43	71
$C^- OM^-$ parent	44	56	53	67
EX^- variant	9	36	11	46
$EX^- C^- OM^-$ variant	21	40	28	51

^a Milliliters per 100 g (R^w) or 100 ml (R^v) of wet spores.

TABLE 5. Comparison of volume percentage of the entire spore occupied by structural compartments within morphotype spores, determined from [^{14}C]glucose permeability and electron microscopy measurements

Morphotype spore	% of entire spore vol		
	Glucose impermeable ^a	Protoplast + cortex ^b	Protoplast ^b
Native parent	57	56	28
C ⁻ OM ⁻ parent	47	66	34
Ex ⁻ variant	89	72	38
EX C OM variant	72	100	60

^a $100 - R^*$ glucose.

^b Calculated from measurements made on electron micrographs of medially thin-sectioned spores.

tent among the four morphotype spores (Table 1) was complicated by two factors that changed as integument layers were successively divested: (i) the weight basis of the water content changed from an entire spore to a cortex-encased protoplast, and (ii) the protoplast volume increased in itself and as a percentage of the entire spore volume (Table 5). Furthermore, the native parent spore of this strain, paradoxically, had a low water content relative to its low heat resistance. Consequently, the values of heat resistance versus water content for the morphotype spores did not fit the correlation line for five other spore types (3).

Apparently, only the resistance parameters that reflect the spore protoplast and cortex are correlated with heat resistance. Thus, among the parameters studied, it was the volume ratio of protoplast to protoplast plus cortex that correlated with heat resistance among the four morphotype spores of *B. megaterium* (Fig. 7). These results were consistent with similar findings in a wide range of other spore species (1, 3, 24; J. E. Algie and L. S. Tisa, Spore Newsllett. 7:20-21, 1981; A. D. Hitchens and R. A. Slepecky, Spore Newsllett. 7:103-104, 1981).

The key parameter of spore resistance should be water content of the protoplast. We have now developed a method for determining protoplast water content by use of lysozyme-susceptible spores and shown that the protoplast water content in three such strains of *B. megaterium* spores is sufficiently low to account for their heat resistance (Beaman et al., in press).

Germination. The response of the four morphotype spores to various germination-initiating agents provided heuristic but incomplete evidence about the role of integument layers in the germination process. Glucose, leucine, and proline might be thought to react primarily in some way with the exosporium, inasmuch as the EX⁻ variant spore (Table 3) and naturally occurring EX⁻ spores (31) did not respond to these agents; however, the addition of isolated exosporium did not evoke a response to these agents by the EX⁻ variant spore. Involvement of the coat and outer membrane complex was also discounted, inasmuch as removal of the complex from the wild-type spore did not affect the response. Involvement of the inner membrane was also discounted, inasmuch as glucose (and therefore the other small germinant molecules) permeated as far as the inner membrane in the C⁻ OM⁻ parent spore but only as far as the outer membrane in the native parent spore (Table 4), yet both of these morphotype spores responded alike to the germinants. The germination-initiating response is dependent on heat activation, and possibly this might be thought to alter spore permeability. In *B. cereus* T spores, however, heat activation does not alter permeability to germinating

agents (alanine and adenosine) or a nongerminating agent (glucose) (7).

Permeability. An outer pericortex membrane apparently exists in addition to the inner pericytoplasm membrane in intact dormant spores. In sectioned *B. megaterium* spores of either the native parent (Fig. 1) (4) or the EX⁻ variant (Fig. 4A), the outer membrane was identified as double-track dark lines complexed between coat layers. Sometimes the peripheral coat layer was separated from the outer membrane so that the membrane could be better distinguished (Fig. 4B). In the chemically divested spores, of course, the outer membrane and coat, of course, were no longer seen. Similarly, both structures are seen to be defective or absent in lysozyme-susceptible mutant spores (10; Beaman, et al., in press).

What we termed the outer membrane may correspond to what Aronson and Fitz-James (2) have termed a double-track or pitted coat layer in sectioned and freeze-etch preparations of *B. cereus* and *B. megaterium* spores. The ordered lattice appearance of the pitted layer surface is not like that usually seen in a bacterial membrane; however, the pitted layer in the dormant spore might be in a crystalline state and resemble the ordered lattice occasionally seen in protoplast membranes (5). Alternatively, the outer membrane may be a different structure than the pitted layer, complexed in the coat, and not seen by freeze-etching because it does not usually cause a fracture plane in the dormant spore (35). Whatever the explanation, the morphological evidence perhaps is less compelling than the biochemical and functional evidence for the existence of an outer membrane in the dormant spore.

Biochemical evidence suggesting the existence of an outer membrane in the dormant spore of *B. megaterium* has been presented and discussed by Crafts-Lighty and Ellar (12), mainly the occurrence of cytochromes, electron transport enzymes and polypeptides in isolated outer integument.

Functional evidence indicates that an outer membrane not only exists but is intact and serves as a permeability barrier in the dormant spore. Rode et al. (32) have shown that, in unfixed spores of *B. megaterium*, methacrylate solution permeates the coat but not beyond a sharply delineated boundary at the juncture of the coat with the cortex; however, the spore treated with a membrane-disrupting fixative no longer possesses this permeability barrier. Another line of evidence for the existence of an outer permeability barrier in the dormant spore has been provided by Carstensen et al. (9), whose dielectric measurements indicate that the cortex is surrounded by a thin outer membrane which insulates against the passage of mobile ions in an electric field. The third and most significant line of evidence for a functioning outer membrane has been provided by permeability studies with three species of spores in which the glucose-impermeable volume corresponds to the physical volume occupied by the sporoplast (essentially the protoplast plus cortex) rather than by the protoplast alone (19). These findings were now confirmed by the permeability results with the native parent and EX⁻ variant spores of *B. megaterium* QM-B1551 (Table 5).

Altogether, the evidence thus indicates the existence in the dormant spore of an intact outer membrane (or possibly a membrane-like layer of the coat) functioning as a permeability barrier to small molecules. Only when the outer membrane is defective or removed does the inner membrane become the primary permeability barrier.

Exosporium morphotypes of *B. megaterium* spores. The parent and variant spores of strain QM-B1551 used in this study are representative of similar morphotype spores with

and without exosporium that occur naturally among *B. megaterium* strains. Recognition of the two different morphotype spores was made independently by two laboratories in 1959. Tomesik and Baumann-Grace (37) used light microscopy to detect an extraperipheral structure (which they termed exosporium) in more than half of 36 strains, by use of negative staining and homologous spore antiserum. Fitz-James and Young (15) used electron microscopy to show that spores of two strains, but not two others, possess an extraperipheral structure (which they termed outer coat) which is thick, loose fitting, and shaped like the hull of an English walnut. This morphological distinction, based on the presence or absence of an extraperipheral structure, was confirmed by Rode (31) and now by us (Fig. 1 and 2A versus Fig. 4 and 2B). Also, Gibson and Gordon, in *Bergey's Manual* (23), recognized two types of *B. megaterium* spores distinguished by fuchsin staining the periphery or not; Fitz-James and Young (15) similarly had distinguished the two types by crystal-violet staining.

Subsequent investigation by Beaman et al. (4) revealed a third morphotype spore among the antiserum-reacting spores studied by Tomesik and Baumann-Grace (37): the spores of strain Mg19 were shown by electron microscopy to contain a typical exosporium with a thick, hairlike nap, unlike the atypical exosporium of strain QM-B1551. Nadirova and Aleksandrushkina (30) also observed three different types of spore surfaces in 10 *B. megaterium* strains.

Altogether, three morphotypes of spores thus can be distinguished among *B. megaterium* strains: one type (exemplified by strain Mg19) contains a typical exosporium like that of *B. cereus*, a second type (exemplified by strain QM-B1551) contains an atypical exosporium, and a third type (exemplified by strain Texas and our variant strain) does not contain either type of exosporium.

Exosporium terminology. Evidence and historical precedence suggest that the extraperipheral structure on spores of *B. megaterium* strain QM-B1551 and similar morphotype strains should be termed exosporium rather than coat, hull, covering, or other nonspecific terms. Unlike coat, this atypical exosporium (as well as typical exosporium) is loose fitting, has apical openings, and is translucent when unstained (Fig. 1 and 2A). Exosporium differs chemically from coat in not being solubilized by alkaline sodium dodecyl sulfate and dithiothreitol (Fig. 3) (2, 26), 1 N NaOH, or hot trichloroacetic acid (15). Furthermore, spores with exosporium are rich in phosphorus (15) and phospholipids (6), including diphosphatidylglycerol, which is the only phospholipid in exosporium isolated from *B. cereus* (28); in contrast, only homogeneous protein characterizes coat (2). Also, the outer peripheral layer (exosporium) differs from the inner layer (coat) of *B. megaterium* and *B. cereus* spores in the mineral matter remaining after microincineration (36).

Historically, de Bary in 1885 (13) first described and Flügge in 1886 (16) first used the term exosporium for the extraperipheral layer of bacterial spores. Tomesik and Baumann-Grace in 1959 (37) kept the term exosporium for *B. megaterium* spores instead of inventing a new expression to avoid increasing the confusion in the nomenclature of the various spore layers. So now should we.

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THE GENETICS OF BACTERIAL SPORE GERMINATION

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INTRODUCTION

Microorganisms in the natural world are likely to spend long periods in
conditions that do not favor rapid growth, interspersed with periods of rapid

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multiplication when a localized supply of nutrients becomes available. Most of the known gram-positive endospore-forming bacteria are soil organisms (86) and are therefore subject to particularly wide fluctuations in environmental conditions. Spore formation in response to nutrient limitation serves as a strategy for survival; its success depends on an efficient germination mechanism to effect the breakage of dormancy, so that metabolic activity and consequent vegetative growth can resume in a favorable environment.

The dormancy and resistance characteristics of the bacterial spore are exceptional for a living cell; it is resistant to extremes of temperature and pH, to desiccation, UV irradiation, enzyme action, and organic chemicals, and may remain dormant for long periods (30). This behavior is achieved by considerable structural specialization, elaborated during an extended period of forespore development within a mother cell. On exposure to relatively low concentrations of particular germinative chemicals, the spores rapidly lose the specialized structural characteristics that contribute to resistance and dormancy (29). An interesting paradox is that the endospore, generally insensitive to gross changes in external conditions, contains a germination apparatus that can survive a considerable degree of environmental insult and yet be sensitive to the presence in the environment of relatively low levels of stimulating germinants.

The process of conversion from bacterial endospore to vegetative cell in response to germinants can be divided into two phases. Germination involves the loss of the spore's dormancy and resistance properties and the reactivation of metabolism (84). The subsequent period in which RNA, protein, and DNA synthesis resume, leading to cell growth and eventual division, is termed *outgrowth* (92). Although the two processes normally form a continuum, outgrowth is distinguished by its sensitivity to inhibitors of macromolecular synthesis and strictly depends on the completion of germination.

The cycle of spore formation and germination in *Bacillus* spp. is a developmental process that has been particularly amenable to analysis; sporulation can be induced in a relatively synchronous fashion in liquid culture and is completed within seven hours. The identification, location, and analysis of developmental genes in *Bacillus subtilis* has been aided by well established transduction and transformation systems and the exploitation of the cloning, sequencing, and expression procedures of contemporary molecular genetics. This review aims to discuss how molecular genetics may help clarify the nature and precise molecular function of the spore's germination apparatus.

SPORE FORMATION AND STRUCTURE

Because spore germination is an integral part of the cycle, a brief introduction of aspects of spore formation and spore structure as well as a description of

the physiology of germination is necessary. A number of well-considered and relatively recent reviews provide more detailed accounts of these subjects. The relationship between the dormancy and resistance properties (4, 30, 50, 107) of the bacterial spore and its structure (106) has been extensively explored. Detailed morphological and biochemical changes occurring during spore formation have been studied in synchronized cultures (25, 114); the phenotype of sporulation (*spo*) mutants blocked at different stages in development suggests that the developmental process involves an ordered and dependent sequence of events (69, 71). Analysis of the nucleotide sequence and regulation of cloned sporulation genes is deepening our understanding of the regulatory elements controlling developmental pathways in the sporulating cell (58, 59). An extensive description of the spore germination response (29) preceded more recent reviews of the biochemical and physiological changes occurring during germination (24, 50, 83). The biochemistry (84, 92) and genetics (26, 71) of outgrowth have also been reviewed.

The pattern of morphological and biochemical changes occurring during sporulation and the resulting spore structure are similar in all the endospore-forming bacteria, justifying a composite picture of the process generated from data obtained in different species. Such a picture can also be used for the major physiological changes during germination, although the effectiveness of particular chemicals in triggering germination varies greatly between species.

Because the spore responds to specific germinants by losing its dormancy properties before macromolecular synthesis resumes, the germination apparatus must be already present as an integral part of the mature spore, and is likely to be synthesized and assembled during sporulation. Regulation of synthesis of spore germination proteins, in terms of timing and localization, is therefore intimately associated with the morphology and control circuits of the developing spore. The following brief summary concentrates on aspects most relevant to spore structure and germination.

Sporulation has been divided into six morphological stages: 0, II, III, IV, V, and VI, on the basis of electron microscopy. A description of biochemical marker events associated with each stage further elaborates them. Following initiation (Stage 0), the first morphological change observed (Stage II) is a specialized cell division, in which a septum develops toward one pole of the cell. This septum is unusual in that cell wall material is not then accumulated between the two cell compartments; instead, the membrane of the larger compartment continues to develop, engulfing the smaller compartment so that a cell *within a cell* structure is generated (Stage III). The inner, or forespore, compartment is bounded by its membrane, which is surrounded in turn by a second membrane of opposite polarity (17). Peptidoglycan is deposited between these two membranes during stage IV; a specialized cortex type is

synthesized by the outer (mother cell) membrane and normal vegetative type by the inner (forespore) membrane (95), forming the *germ cell wall*, which is retained on germination. Calcium ions are taken up into the mother cell by active transport and incorporated into the inner cell compartment by facilitated diffusion (17). Dipicolinate (DPA) is synthesized in the mother cell and accumulated in the forespore. Coat proteins are synthesized in the mother cell and eventually assembled round the outer surface of the spore at stage V. The forespore matures, becoming increasingly phase-bright and developing progressively greater heat resistance (stage VI). The germination characteristics of the mature spore are established at late stages of spore formation (13). The mother cell compartment, which contributed materials to the developing spore, eventually lyses to release the mature spore (stage VII).

The products of a large number of *spo* genes are required for this complex developmental process. Many *spo* genes have now been cloned, and the dependence relationships determined for some (59). Expression of stage II genes depends on the activity of stage 0 genes. After septation, different genes are expressed in the two compartments as the pathways of dependence for mother cell and forespore diverge. Of the four sporulation-specific sigma factors so far defined, at least sigma G and K are active only in forespore and mother cell respectively (91, 94).

The final product of the developmental process is a mature, resistant spore. The general consensus is that its heat resistance is a consequence of the low water activity in the spore core, but a variety of models exist to explain how this is achieved (27). The spore cortex appears to play a role in the dehydration of the core by exerting physical or osmotic pressure, or possibly both (30, 107). The role of calcium dipicolinate in heat resistance is less clear, but it may act as a secondary stabilizing agent (107). The small acid-soluble proteins (SASP) α and β , synthesized in the forespore and present at high concentrations in the spore core, contribute to the spore's high UV resistance—mutants lacking both these proteins are sensitive to UV (60). The spore coat layers are not essential for dormancy or heat resistance, but are important in the resistance of the spore to enzymic attack, e.g. by lysozyme.

The spore contains two membrane systems; the inner, which originated as the forespore membrane, bounds the cellular compartment, and the outer, which represents the residuum of the outer forespore membrane, lies immediately underneath the coat layers. An outer membrane is not usually visible in electron micrographs of sections of spores, but components such as cytochromes and electron transport chain enzymes are found in outer integument fractions of spores (9). Whether the outer membrane remains as a lipid bilayer that constitutes a permeability barrier is not certain but is important to an understanding of the action of germinants. Experiments on *Bacillus megaterium* QMB1551 spores indicate the presence of a membranelike layer

on the inner surface of the spore coat, which functions as a permeability barrier to glucose and ribose (52). If this layer is removed, the inner membrane becomes the permeability barrier. This membrane separates two compartments, core and cortex, that are hydrated to different extents and is thought to be in a semicrystalline state, probably complexed with calcium ions. It returns to a more fluid state on germination, along with the release of Ca^{++} from the spore (89).

SPORE GERMINATION

Spores of many bacteria are extremely slow to respond to germinants and depend on activation by brief sublethal heating or longer storage at low temperature, which may increase spore permeability (51). For some spores, including those of *B. subtilis*, activation is inessential but increases the rate of germination.

L-alanine (L-ala) is a common, though not universal, germinant (22, 29, 36, 82), whose activity is competitively inhibited by the D isomer (36). A variety of alanine analogues, some nonmetabolizable, can substitute for L-ala as germinant (49, 77, 109). Some bacilli require a riboside, typically inosine, as well as alanine for maximal germination, while others germinate in sugars plus inorganic ions or in response to ions alone (22, 55). Some spores can germinate in more than one type of germinant; *B. megaterium* QMB1551, for example, will respond to glucose, proline, or inorganic ions (55), whereas *B. subtilis* germinates in a combination of asparagine, glucose, fructose, and KCl (AGFK) as well as in L-ala (108).

Germinants are thought to act as triggers (33) because their interaction with the spore leads rapidly to a series of major changes in its structure and physiology. Biochemical changes accompanying germination have mainly been monitored using species such as *B. megaterium* and *Bacillus cereus*, whose spores respond to germinant more rapidly and synchronously than those of *B. subtilis*. The pattern in *B. subtilis* is generally similar, but the order of events is harder to distinguish because of the less synchronous response (63, 99).

After a brief period of exposure to germinant, the spore is committed to germinate, even if the germinant is removed. In *B. megaterium* KM (90), at least 50% of spores were committed within three minutes. This commitment precedes detectable changes in the spore that include, in approximate temporal order (31, 56, 82), loss of heat resistance, K^{+} and Zn^{++} fluxes (16, 48), release of Ca^{++} and DPA from the core, hydrolysis of cortex peptidoglycan (23, 47), rehydration of the core protoplast, and the resumption of metabolic activity (12, 74, 80, 81). Nicks appear in the spore coat at an early stage in germination (79), but some coat layers persist. Hexosamine-containing frag-

ments are released and the core swells to occupy the space previously occupied by cortex (79). The spore protoplast, bounded by membrane and germ cell wall, must increase its surface area considerably at this time. As the core rehydrates, the spores change progressively from phase-bright to phase-grey and then to phase-dark; the alteration in light-scattering behavior of a suspension of spores results in a loss of approximately 50% of the suspension's optical density (OD) over the germination period. In *B. megaterium* KM these postcommitment stages take six to eight minutes (47). Turnover of preexisting lipid, RNA, and protein occurs at later stages of germination (84); best studied is the degradation of SASPs during late stages of germination to generate amino acids for the resumption of protein synthesis (85). Figure 1 outlines the events in germination.

Inhibitors that prevent triggering include D-alanine, which competes with L-alanine, presumably by binding nonproductively to the receptor; alcohols

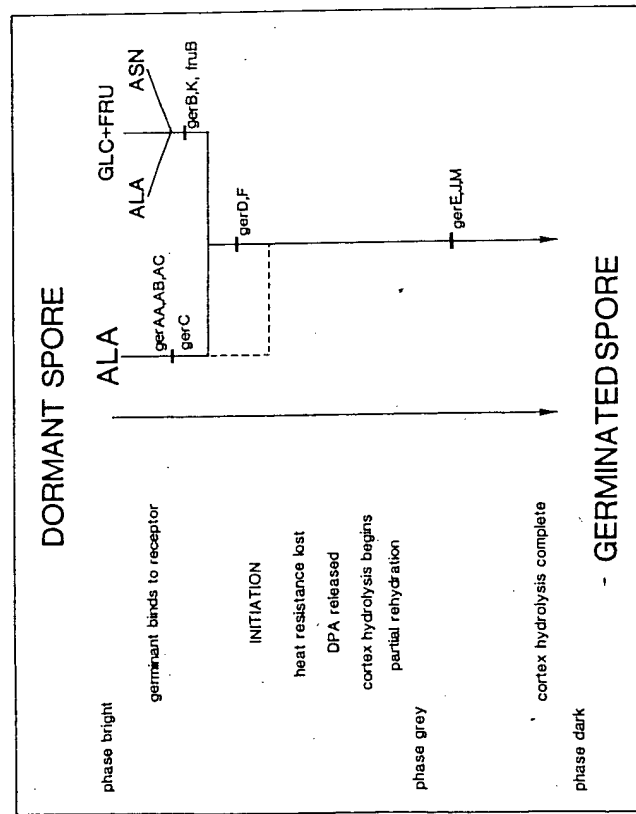


Figure 1 Events occurring during germination of *B. subtilis* spores. The sequence of events indicated is approximate, particularly with respect to the relative order of the onset of cortex hydrolysis and release of DPA. The molecular event(s) represented by initiation have not yet been identified. The scheme on the right represents the mutational blocks in germination that have been identified; the dotted line indicates that the defect in alanine germination in *gerD* and *gerF* mutants can be bypassed to some degree.

[suggesting that the receptor is in a hydrophobic environment (111)]; and methyl anthranilate, which may inhibit in a specific manner (72) or because of its hydrophobicity. Several inhibitors differentially affect the two germination systems in *B. subtilis* (99); azide inhibits only AGFK germination and phenyl methyl sulphonyl fluoride (PMSF) inhibits only L-alanine germination. Cyanide and arsenate, both inhibitors of electron transport, do not inhibit triggering of either pathway, but protease activity may be important as both alanine and AGFK germination are sensitive to tosyl arginine methyl ester (TAME). Mercuric chloride can block germination at an intermediate stage (56); for *B. subtilis* spores, triggering in AGFK is sensitive to low concentrations of the inhibitor, whereas in L-alanine the most sensitive stage is postcommitment (99). The onset of outgrowth is marked by a resumption of RNA and protein synthesis, then of DNA replication and eventually cell division (84). The expanding protoplast emerges through the residual coat layers and elongates to form a vegetative cell. Outgrowth represents a distinct phase of development, as six classes of mutant (*outA-F*) that are specifically temperature sensitive during this stage but not during vegetative growth have been described (26). The *outB* gene and other genes expressed specifically during this period have been cloned (21, 28), but the regulation of gene expression during outgrowth is only beginning to be explored (1).

Germination of spores can be monitored in a number of ways. Loss of OD or loss of heat resistance in suspensions of germinating spores can be determined, and phase-darkening provides a direct but qualitative measurement of spore germination. A *uvr ssp* mutant, deficient in the repair of UV irradiated spore DNA and whose spores are therefore more sensitive to UV when dormant than when germinated, was used to measure the kinetics of germination at limiting alanine concentrations (39); germination may require that three molecules of alanine interact with the spore.

Genetic studies of spore germination have depended on the exploitation of a plate test to distinguish *Ger*⁺ and *Ger*⁻ colonies, so permitting the rapid scoring of large numbers of recombinants (54, 97). Spore-containing colonies, in which any remaining vegetative cells have been killed by heat or by exposure to chloroform vapor, are overlaid with agar containing germinants and 2,3,5-triphenyltetrazolium chloride. Wild-type spores germinate and resume dehydrogenase-linked metabolism, reducing the tetrazolium to an insoluble formazan that stains the colony red (TZM-Red). Mutants that do not germinate remain unstained (TZM-White).

No one individual parameter is an adequate description of the germination behavior of a spore preparation. The OD fall, for example, represents a summation of the germination response of the entire population and its profile will reflect the asynchrony of germination. A slower germination rate may reflect a delayed response by individual spores to germinants. A second

problem is that several phases of germination contribute to the loss of OD. A similar profile would be obtained whether half the spores in a population germinated completely or all the spores germinated to an intermediate extent. Measurement of an early event, such as loss of heat resistance, is therefore required to distinguish between these possible interpretations.

The tetrazolium plate test also has its limitations, as germination mutants defective in the AGFK pathway are TZM-Red, but a modification involving germination of spores on filters (38) allows these mutants to be distinguished from the wild-type.

GERMINATION GENES AND THEIR ANALYSIS

Characterization of Mutants

Mutants affecting germination in a variety of *Bacillus* and *Clostridium* spp. have been reported (8, 98, 105, 110), but researchers have only been able to analyze genetically those generated in *B. subtilis*.

The resistance properties of dormant spores has been exploited in the isolation of *B. subtilis* germination (*ger*) mutants. A spore suspension is incubated with germinant and germinated spores killed by heating or exposure to chloroform, thus enriching the suspension with mutant spores that had not germinated (65, 97). Because of the asynchrony of germination of wild-type spores, the suspension is generally taken through several cycles of enrichment before remaining ungerminated spores are recovered by incubating overnight on a rich agar medium. Spores prepared from the resulting colonies are then screened for germination abnormalities by measuring changes in OD₅₈₀ of spore suspensions in response to germinants and/or by testing the behavior of spore-containing colonies in the tetrazolium plate test. The strong correlation between TZM-White or TZM-Pink colony color and a measurable defect in germination of the spore suspension simplifies the task of screening for mutants amongst the survivors.

As the mutants are obtained from the spore form by germination, any completely nongerminating mutant would not be recovered. Many mutants are selected as conditional (temperature-sensitive or germinant-specific, depending on the enrichment procedure adopted). Other isolates that have a more general defect in germination are leaky in that a small proportion of the spores germinate, outgrow, and form colonies on rich agar.

Transposon Tn917 (115) has three advantages for mutagenesis of *ger* genes; total inactivation of a gene can be assured, the mutant allele can be selected in genetic crosses, and, using modified transposons (116), DNA adjacent to the point of insertion can be cloned directly. Transposon insertion mutants were first screened using the tetrazolium plate test to identify likely germination mutants before measuring germination in spore suspensions (78, 113).

Other characteristics of *ger* mutant spores, such as the extent of phase darkening, release of DPA, and loss of hexosamine during germination, have been studied; some mutants were also found to have sporulation abnormalities. The germination mutants can be grouped loosely according to their phenotype. This classification is reinforced genetically; similar phenotypes result from *ger* mutations at a similar location on the genetic map (65, 66, 71, 88), which identifies a number of genes with a role in germination (Table 1). Mutants isolated elsewhere that are also defective in germination are included in Table 1.

Spores of two classes of mutant, *gerA* and *gerC*, are defective specifically in their response to L-alanine but germinate normally in AGFK. The converse phenotype is seen in *gerB*, *gerK*, and *fruB* mutants, in which germination is normal in L-alanine but is not stimulated by AGFK. This difference suggests that the spore has two separate systems for detecting the alternative germinative stimuli. The *gerD* and *gerF* mutants are defective in their response to L-alanine.

Table 1 Germination-associated loci

Locus and map position	Germination response L-alanine	AGFK	Additional information ^a
<i>gerA</i> (289°)	—	+++	Encodes three membrane-associated polypeptides (110). Alanine receptor complex?
<i>gerB</i> (314°)	+++	—	Gene cloned. Response to sugars defective (65).
<i>gerC</i> (201°)	ts	+++	Also affects vegetative growth. Cloned and sequenced; maps in operon of three genes (112).
<i>gerD</i> (16°)	++	—	Defect more extreme in AGFK (104); encodes a single protein (113).
<i>gerE</i> (253°)	P	P	Blocked after loss of heat resistance (63). Encodes a regulator of coat gene expression (11).
<i>gerF</i> (301°)	+	—	Defect more extreme in AGFK (101).
<i>gerG</i> (294°)	+	++	Lacks phosphoglycerate kinase (<i>pgk</i>). Sporulation defect (73).
<i>gerJ</i> (206°)	P	P	Blocked after loss of heat resistance. Late sporulation delay. Cortex defect? (102)
<i>gerK</i> (32°)	+++	—	Spores defective in response to glucose (38).
<i>gerM</i> (251°)	P	P	Single protein, possible lipoprotein. Some cells blocked at stage II (78). Cortex defect?
<i>spoVIA</i> (255°)	+	ND	Spore coat defective (43).
<i>spoVIB</i> (247°)	+	ND	Spore coat defective (44).
<i>spoVIC</i> (294°)	+	ND	Spore coat defective (41).
<i>colT</i> (114°)	+++	—	Spore coat protein mutant; coat structure disturbed (2).
<i>fruB</i> (120°)	+++	—	Fructose-1-P kinase defect (73).

^a Other loci whose status is uncertain include *gerH* (88), *gerI* (probably identical to *gerF* (88)), and *gerL*, which confers resistance to barbitol but may not be germination specific (67). Germinants are L-alanine (L-alanine), and asparagine + fructose + glucose + KCl (AGFK). Symbols indicate: +, full response; ++, intermediate response; +, poor response; —, no response; P, blocked at a late (phase-grey) stage; ND, not determined. Map positions are from (or consistent with) the genetic map of Pigot and Hoch (70).

and AGFK, and probably represent genes whose products have a role in both germination pathways. Mutations in several loci (*gerE*, *gerJ*, *gerM*, *spoVIA*, *B*, *C*, *cofT*) affect both the germination properties of the spore and the overall structure of the cortex or coat. The gene products of these loci have a regulatory or structural role during spore formation so their effects on spore germination may be indirect.

The genetic analysis has identified a number of genes required for germination (Table 1) but is unlikely to have identified all the proteins important in triggering, as mutations in genes essential for vegetative growth would not have been detected, and neither would mutations in genes whose products function in germination but are not absolutely essential for the process.

Genes Involved in the L-Alanine Germination Pathway

GERA The *gerA* mutants (65, 77, 97) are defective in their germination response to L-alanine, L-valine, and cycloleucine but germinate normally in AGFK. Some are temperature-sensitive in alanine germination. The response of spores to L-alanine is blocked at the earliest stages of germination, before commitment, as the spores do not lose heat resistance. All of the *gerA* mutants can utilize alanine as sole carbon source and those tested exhibited normal chemotaxis toward alanine. Spores of *gerA38* or *gerA44* mutants germinate in alanine and related germinants if concentrations higher than those required by the wild-type are employed, and are also altered in the ratio of D- to L-alanine required for competitive inhibition of germination. These characteristics are consistent with the hypothesis that *gerA* encodes a receptor in the spore that binds alanine and transduces the germination stimulus—the putative site of triggering of alanine germination (77). The *gerA* mutants still retain some response to alanine, as they will germinate when it is used in combination with glucose and fructose (65, 97).

Cotransduction frequencies of 70–90% in SPP1-mediated transduction imply that *gerA* maps very close to *cirG*, which encodes fumarase. Lambda clones carrying *cirG* were selected by complementation of an *Escherichia coli* fumarase mutant, and the presence of *gerA* DNA was confirmed by conjugation (64). Subsequent complementation analysis and sequencing of *gerA* (20, 118, 119, 120) revealed an operon of three genes suitably arranged for translational coupling.

The *gerA* genes I, II and III have been renamed *gerAA*, *AB*, and *AC*, respectively, to be consistent with the nomenclature of *spo* genes. The products of all three genes are required for the spore to respond to alanine as sole germinant; the *gerA38* and *gerA44* mutations that confer an altered concentration-dependence map in gene *gerAB* (119). The deduced 480-residue protein product of *gerAA* contains a large central hydrophobic domain of some 250 residues that would be membrane-located, flanked by long

N-terminal and shorter C-terminal hydrophilic domains. Whether the two hydrophilic segments would lie on the same or opposite sides of the membrane is not obvious. The 364-amino acid *gerAB* protein has the hydrophathy profile characteristic of an integral membrane protein, with approximately 10 membrane-spanning helices separated by charged regions likely to be exposed on the surface. The *gerAC* gene encodes a 373-residue protein that is relatively hydrophilic, but has at its N-terminus the potential signal peptide of prelipoprotein. The protein might be transported across a membrane and immobilized at the surface by covalent linkage to lipid.

If the *gerA* proteins do indeed represent the receptor for alanine, the data imply that this receptor would be a membrane-located complex containing at least these three polypeptide subunits.

Analysis of *gerA-lacZ* fusions revealed that the *gerA* operon is switched on two hours after initiation of sporulation (120) in the forespore compartment (19). Expression from the *gerA* promoter can be switched on in vegetative cells by artificial induction of the forespore-specific sigma G factor (19, 68). The *gerA* proteins are therefore probably located in the forespore membrane because this membrane surrounds the cellular compartment in which they are synthesized. Levels of *lacZ* expression directed from the *gerA* promoter were 300-fold lower than those from a *cirG* promoter fusion; the levels of *gerA* proteins are probably correspondingly low (120).

To obtain expression of the *gerA* genes in *E. coli*, they have been fused downstream of the controllable λ PL promoter. Using in vitro expression of this construct and various deletion derivatives, J. McCarvil & E. H. Kemp (unpublished data) have related polypeptides of the predicted size to individual *gerA* genes. Attempts to overexpress these proteins in growing cells have failed, probably because of the proteins' association with the membrane. A translational fusion of the N-terminal domain of *gerAA* to the β -galactosidase protein can, however, be overexpressed and is being used to raise antibodies (A. Moir & E. H. Kemp, unpublished data).

Neither database scans nor direct comparison with the sequence of membrane transport proteins have revealed significant homology between *gerA* polypeptides and any other protein. However, the sequence of part of an open reading frame (*spoVAF*), downstream of the five *spoVA* genes and probably part of the same operon, aligns with and is 30% identical to the *gerAA* protein (J. Errington, personal communication). Information on how far the homology extends must await the cloning of downstream genes. As part of the *spoVA* operon, this *gerAA* homolog is also expressed in the forespore. Could a family of *gerA*-like proteins reside in the spore and what is their role? Are they all membrane located? A frameshift mutation in *spoVAF* does not block sporulation (J. Errington, personal communication), and it will be interesting to see whether the resultant spores germinate normally.

GERC The *gerC* mutants (97) display a temperature-sensitive alanine-specific phenotype. They fail to germinate in alanine at 42°C but do so at lower temperatures. Germination in AGFK is normal. The *gerC* locus was difficult to analyze because *gerC* mutants carried linked mutations that complicated the germination phenotype (65). Strains carrying *gerC58* grow very slowly on minimal agar and barely at all on rich medium and rapidly accumulate suppressing mutations that restore normal growth. The suppressor has not been studied in detail but is not linked to *gerC* in transformation. The suppressed strains sporulate, and spores are germination-defective. Transformation crosses suggest the gene order *gerC-aroB-rpC*, and the *gerC* gene has been cloned in phage 105 by complementing the growth defect in an unsuppressed strain (112, 112a). Sequence studies of *gerC* (M. A. Yazdi & J. P. Curson, unpublished data) have so far identified a cluster of at least three genes located between *mir* and the *aroFBH* operon; the *gerC58* mutation lies in the second or the third gene of the *gerC* operon, but we do not yet know whether other genes in the operon are important in germination. None of the genes so far sequenced would encode a product that is particularly hydrophobic or has any obvious membrane association, nor have any related proteins been detected in database scans. As the *gerC* mutation affects the growth phenotype, it must represent a gene that is active in vegetative growth as well as function during germination. Analysis of this locus is still at an early stage, but *gerC* is likely to encode one or more products that interact with the alanine-stimulated germination system, possibly with the receptor.

Genes Involved in the Alternative (AGFK) Pathway

GERB Spores of *gerB* mutants are defective in AGFK but entirely normal in L-alanine germination (65). Double mutants carrying *gerA* and *gerB* defects do not germinate in alanine plus sugars (A. Moir, unpublished data), demonstrating that the residual response to alanine in the *gerA* mutants is related to the AGFK pathway. The *gerB18* mutation maps on the *cysB*-distal side of *hisA* (65) in the order *graB-tag-gerB* (B. Corfe & D. A. Smith, unpublished data). A set of overlapping lambda clones extending through the *gla-tag* region (which encodes the genes of teichoic acid biosynthesis) have been obtained (61). One of these carries the *gerB* gene and transformation data from subclones reveal that the *gerB15* and *gerB18* mutations lie on opposite sides of a *Bam*HI site, which is approximately 7 kb from *tag* (B. Corfe, personal communication).

GERK As is the case for *gerB*, spores of *gerK* mutants (38) are defective in AGFK and normal in L-alanine. Irie et al (38) proposed that the response to glucose is defective in these mutants. The *gerK* locus maps between *aroI* and

dal (38), not far from the *gdh* (glucose dehydrogenase) gene. The possibility that this enzyme might be necessary for AGFK germination (93) has recently been discounted, as a null mutant of *gdh* generated using reverse genetics germinates normally (R. Ramaley, personal communication), and clones carrying the *gdh* operon do not carry *gerK* (R. Irie, unpublished data).

FRUB Spores of a *fruB* mutant fail to germinate in response to fructose (73). They can still germinate if mannose is substituted for fructose, suggesting that the germination defect is fructose-specific. Because the strain is defective in fructose metabolism, this specificity argues strongly that the metabolism of fructose is important for its role as an auxiliary germinant.

Genes Common to Both Germination Pathways

GERD Mutations in the *gerD* gene (40, 65) map close to the *rpsI* (kasugamycin resistance) gene in the order *cysA-rpsI-gerD* (113) near a cluster of rRNA genes. Spores of *gerD* mutants are defective in their response to both L-alanine and AGFK, and are blocked at an early stage of germination, prior to the loss of heat resistance. Although the spores do not germinate in AGFK, germination rates in L-alanine can be significant, depending on which medium the spores were produced and the ionic conditions during germination (104). The differential response to alanine and AGFK is also seen in a *gerD* null (Tn917) mutant (113); presumably the process blocked is critical to AGFK germination but can be bypassed in L-alanine germination. The effects of ions and sporulation medium were also observed, albeit to a lesser degree, for wild type spores germinating in alanine, suggesting that their stimulation of low germination rates was a general effect. The residual alanine response is *gerA* dependent; it was absent in a *gerA gerD* double mutant.

The *gerD* gene encodes a 185-residue protein that would be hydrophilic, except for a hydrophobic N-terminal region that could represent a signal sequence or a membrane-anchoring helix (113). This locus is probably monocistronic, and preliminary experiments suggest that it is expressed from two hours after the initiation of sporulation (113).

GERF Spores of *gerF* mutants are defective in germination in both alanine and AGFK (65), and are blocked before loss of heat resistance. Like the *gerD* mutants, they are particularly defective in AGFK, but the alanine defect is more marked than in *gerD* mutants and is significantly overcome only if both sugars and K⁺ are added (101). The *gerF* locus is close to *hisA* (approximately 57% cotransduced by SPP1) but has not yet been cloned.

GERG (PGK) This gene encodes phosphoglycerate kinase; spores of a *pgk* mutant germinate very poorly in L-alanine and somewhat slowly in AGFK

(73). The strain does not sporulate efficiently (73; A Moir, unpublished data). Whether the germination defect is an indirect effect of the formation of structurally abnormal spores is not clear.

Genes Involved in Spore Structure

The following mutants were isolated on the basis of the germination properties of their spores, but their spores are also altered in either cortex or coat.

GERE. Spores mutant in this gene germinate to an intermediate stage, losing heat resistance and DPA, but exhibiting incomplete hydrolysis of the spore cortex. They have a severe defect in coat structure (63); many of the normal proteins are absent (45). As a result they are sensitive to lysozyme, which cannot penetrate the wild-type coat. Presumably the coat is also permeable to any soluble enzyme in the cortex that is free to diffuse, and some proteins may be missing from the mature *gerE* mutant spore for this reason. Other characteristics of the germination response suggest that the initial interaction with alanine is unaltered, as the spores lose heat resistance with kinetics that are, if anything, more rapid than wild-type (63). Thus, the initial response to germinants does not require that the spores have an intact coat, and the alanine germination receptor is therefore not likely to be in the coat. The mutant also lacks proteases normally associated with the developing spore (42); whether any of these are important in germination is not known. The *gerE* gene has been cloned (34, 42) and encodes an 8.5-kd protein that is strongly homologous to the DNA-binding domain of the regulatory components of sensor-regulator pairs (10, 35). It acts as both a positive and a negative regulator of coat gene expression, as the *gerE36* nonsense mutation prevents expression of *cotB* and *cotC* but leads to overexpression of *cotA* and *cotT* (2, 11). The *gerE* gene is expressed from about four hours after the initiation of sporulation (11, 18) and is transcribed by sigma K, whose structural gene is formed by a gene rearrangement in the mother cell compartment (53, 91). The transcriptional activity of the *gerE* gene in *spo* mutant backgrounds suggests that *gerE* is regulated by genes required for expression in the mother cell, but that its expression is also linked to morphological changes in the spore (11).

GERJ. Like *gerE* mutants, *gerJ* mutant spores are blocked at the phase-grey stage in L-alanine and AGFK. The spores have no detectable defect in their coat structure, but are somewhat less heat resistant than wild-type spores (103), and the resistance properties of the forespore develop later than normal during sporulation (100, 102). A study of penicillin-binding proteins (PBPs) in the *gerJ* mutant revealed a corresponding delay in synthesis of a sporulation specific PBP, PBP5* (102). The *gerJ* gene is likely, therefore, to represent another regulatory locus, perhaps concerned with cortex biosynthesis. A *lac* fusion to *gerJ* generated using a modified Tn917 reveals that the gene

switches on one and a half hours after the initiation of sporulation (102). The incomplete germination of *gerJ* mutant spores may result from either an altered spore cortex not responding fully to the normal lytic enzymes or the absence of some protein, whose expression is *gerJ*-regulated, required for cortex hydrolysis during germination. DNA adjacent to a Tn917 insertion in *gerJ* has been recovered, and clones carrying the intact *gerJ* gene obtained from λ libraries are being sequenced (R. Warburg, H. Chicamane, O. J. Marvik, and H. O. Halvorson, personal communication).

GERM. The *gerM* locus, defined by Tn917 mutation, maps close to *gerE* but is separated from it by 2.1 kb of DNA (78). The germination response of *gerM* spores to L-alanine and to AGFK, although initiated normally, is blocked at an intermediate, phase-grey stage; cortex hydrolysis, although incomplete, appears to progress further than it does in a *gerJ* mutant (78). The *gerM* strain is oligosporogenous; many cells are blocked at stage II, forming aberrant structures in which multiple polar septa have been initiated and peptidoglycan accumulated between the membranes of the septum. Cells that escape this fate and compartmentalize successfully form spores with an aberrant cortex, as judged by their altered heat resistance. The *gerM* gene, like *gerJ*, is expressed from about one and a half hours after the initiation of sporulation. It encodes a 22-kd protein with a potential lipoprotein signal sequence, suggesting that it is anchored at the surface of a membrane (87). The protein is important for correct septal development and probably also for subsequent cortex synthesis; the oligosporogenous phenotype of a null mutant implies that its role in septum formation can be partially met by some other protein.

SPOVI. A search for germination mutants with coat defects yielded three classes, *spoVIA*, *B*, and *C*, whose spores are altered in their response to L-alanine. AGFK germination was not measured (41, 43, 44). A fraction of *spoVIA* and *B* mutant spores lose resistance to toluene and become phase-dark; the others remain phase-bright. This behavior is distinct from that of *gerE* mutants, whose spores are blocked in later stages of germination and all become phase-grey. The *spoVIB* and *C* products are probably regulatory because they affect the time of synthesis of coat proteins and consequently disturb normal coat deposition patterns (41, 44).

Disappointingly, all the mutants so far isolated that affect later stages of germination produce structurally defective spores—novel selection procedures may, therefore, be required to isolate mutants defective in specific components of the degradative cascade.

Spore Mutants Constructed by Reverse Genetics

The spore coat of *B. subtilis* contains approximately a dozen major polypeptides (46). Genes encoding polypeptides of 65 kd (*cotA*), 59 kd

(*cotB*), 12 kd (*cotC*), 11 kd (*cotD*), 24 kd (*cotE*), and 12.5 kd processed to 7.8 kd (*cotF*) have been cloned (2, 14, 117). Insertion or deletion mutations in these genes have been constructed in vitro and returned to the *B. subtilis* chromosome. Spores of mutants in *cotA* to *E* germinate normally or nearly so, although *cotD* and *E* spores may germinate marginally more slowly in alanine than do those of wild-type (117; R. L. Sammons, personal communication). The *cotF* protein may have a scaffolding role because mutants appear to be pleiotropic; they have a disturbed coat structure rather than one lacking a single protein component. Germination of *cotT* mutant spores is normal in L-alanine but is very slow in AGFK (N. Bourne & A. I. Aronson, personal communication). A mutant that overproduces *cotT* precursor and deposits large amounts of the unprocessed protein on the surface of the spore germinates slowly in both germinants, probably as a result of decreased spore permeability (2). To know whether *cotA-D* mutants germinate normally in AGFK would be interesting; so far, only *cotE* spores have been tested and found to have no defect in AGFK germination (R. L. Sammons, unpublished data).

A mutation has been introduced by reverse genetics into a gene encoding BBP5 (96). Spores of this strain are less heat resistant and their cortex may be less extensively crosslinked; their germination characteristics have not yet been reported.

The genes encoding small, acid-soluble proteins (SASPs) have been cloned and the chromosomal copies inactivated (85). Spores lacking SASP α and β germinate normally, although they outgrow slowly (60).

MODELS OF SPORE GERMINATION

Various models for the triggering of germination have been proposed and the evidence for them has been extensively discussed in other reviews of germination (24, 50). Germinant molecules interact in a specific manner with the spore, presumably with a spore protein. Models that invoke metabolism of the germinant (72, 73) predict that this interaction leads to enzymic conversion of the germinant or, in the case of an ionic germinant, of some internal metabolite. Models that propose that metabolism is not involved (76, 80, 81) imply that the germinant acts allosterically on a receptor protein. Various possible mechanisms for the transduction of the stimulating signal have been suggested. Germinant binding may alter membrane permeability, possibly via the activation of an ion transporter or channel in the membrane (50, 62). Inhibitor studies have implicated a proteolytic activity in germination (5, 6), which may in turn activate a cortex lytic enzyme (23) by releasing it from a cortex-bound proform. A 10.2-kd protein that might represent a receptor for proline has been detected in the membrane fraction of *B. megaterium* QMB1551 spores (75). This protein can be photo-affinity labelled by a

proline analogue in wild-type spores but is not labelled in a proline-defective germination mutant; unfortunately the protein has not been purified.

Mutational analysis does not suggest that metabolism is necessary for the triggering of germination by alanine. This observation is consistent with isotopic labelling studies of alanine-triggered germination in *B. megaterium* (80, 81), which demonstrated that no significant metabolism occurred before commitment. Instead, the genetic studies have implicated three spore proteins encoded by the *gerA* operon. These proteins would be located in the inner membrane of the spore, presumably as a complex, and associated domains or subunits would extend on at least one and probably both sides of the membrane. The genes are expressed at a very low level and presumably the protein complexes are not abundant in the spore membrane. Because of these findings, we propose that alanine acts as a germinant by penetrating the spore as far as the outer surface of the inner membrane, where it interacts with a receptor complex; this interaction results in the transduction of the germination stimulus, possibly via the *gerC* protein, to initiate a cascade of subsequent hydrolytic reactions. A biochemical approach will be required to establish whether the proteins associate in a membrane complex as predicted and whether the complex can bind alanine; a clarification of the function of the *gerA* and *gerC* proteins is the key to an understanding of alanine-stimulated germination.

The response of the *B. subtilis* spore to L-alanine is complicated by a second alanine-sensitive system independent of *gerA*. Spores of a *gerA* mutant germinate normally in AGFK, and they also germinate if glucose and fructose are included with L-alanine. This system depends on *gerB* and *gerK* function, and, therefore, alanine acts in this case in an analogous manner to asparagine, possibly by interacting with a different receptor that cannot initiate a germination response on its own (Figure 1).

The germination process, in response to AGFK is clearly distinguishable from that in L-alanine. The systems are both sensitive to a protease inhibitor, but differ in their sensitivity to other inhibitors. The products of *gerB*, *gerK*, and *frtB* are essential for the response to AGFK but have no role in the major L-alanine germination pathway. Two other loci (*gerD* and *gerF*) have been identified that have a minor role in the L-alanine pathway but are essential for the AGFK route. At the moment, we cannot rule out the possibility that one or more of the *gerB*, *D*, *F*, or *K* proteins could act indirectly, if the gene affected some aspect of spore structure. Temperature-sensitive germination mutants in these genes, if isolated, would confirm whether the gene products participate directly in the germination process. Interestingly, a structural mutant, *cotT*, is defective only in the AGFK pathway of germination, which may imply that some component of AGFK interacts with the coat or adjacent layer to play its part in the initiation of germination. The integrity of the coat is not essential, however, as triggering by AGFK proceeds normally in a *gerE* mutant (65).

Mutations in *gerE*, *J*, or *M* genes result in the formation of defective spores that initiate a germination response but cannot complete the process. This inability may result from the failure to express a protein important for these late stages, a failure to assemble such a protein in the correct location, or an alteration in the target of the protein (such as the cortex) so that the protein can no longer act. These genes are extremely interesting from the point of view of sporulation control and morphogenesis because they define genes concerned with the synthesis of coat (*gerE*, *spoVIA*, *B*, and *C*) and cortex (*gerJ* and *M*).

One recent hypothesis (23) proposes that activation of a cortex-bound lytic enzyme is a crucial and early stage in alanine-stimulated germination of *B. megaterium* KM. In this case, as in any in which a particular protein is implicated in the germination process, a directed approach to clone the gene, inactivate it, and then examine the phenotype of the mutant is essential.

CONCLUSIONS

Spore germination has been difficult to study because it involves extremely rapid physiological responses in a cell whose structure is biochemically intractable. Models for the mechanism of germination triggering in response to low concentrations of germinant have been based on physiological and inhibitor studies; the strength of the genetic approach is that it reverses the argument by first defining genes whose function is important in the progress of germination, focussing subsequent biochemical analysis on components with a proven role. By cloning and sequencing these genes and studying their regulation, we are learning more of the nature of their protein products. Deciphering the function of these proteins during germination is, however, more difficult. Currently, we have identified proteins of known importance but unknown biochemical function—the *gerA* proteins—and proteins of known function, such as cortex lytic enzymes, important in cortex hydrolysis but whose essential nature for germination is not proven. An integrated approach involving both genetics and biochemistry is essential for further progress. The mutational analysis has set the agenda for the next stage in analysis of spore germination but a satisfactory explanation of the process in molecular terms is still some way off.

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The genetic analysis of bacterial spore germination

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1. INTRODUCTION

The formation of a bacterial endospore is a complex and sophisticatedly-regulated process of structural differentiation, responsible for the resistance and dormancy properties of the spore (Errington 1993). However, the success of this strategy for survival is dependent on the presence in the spore of an efficient mechanism for returning the organism to the vegetative state, allowing growth and multiplication when nutrients are available.

Despite the spore being insensitive to environmental insult, it must be able to respond to *particular* external chemical stimuli by germinating, losing the spore structural properties that confer dormancy and resistance (Gould 1969; Setlow 1981; Moir 1992). Germination, which may be defined as the loss of spore resistance properties, is followed by a period of outgrowth, when biosynthetic activity is resumed and an actively dividing rod-shaped cell is regenerated (Setlow 1984).

There is a general assumption that the molecular events in germination, as in sporulation, will be similar in nature across the range of endospore-formers. Certainly the gross morphological and biochemical changes and changes in spore structure at germination are common to all species, although the natures of the chemicals that are effective as germinants differ. Although the particularly thick coat and less synchronous germination response of spores of *Bacillus subtilis* 168 has meant that it is not the organism of choice for biochemical studies, it is the only spore former in which sophisticated genetic analysis is possible.

2. HOW MIGHT GERMINANTS ACT?

There is an extensive literature describing the response of spores of a variety of species to particular germinants (Gould 1969; Smoor and Pierson 1982; Moir 1992) and the structural and biochemical changes occurring during germination (Levinson and Hyatt 1966; Gould and Dring 1972; Scott *et al.* 1978; Foster and Johnstone 1989; Venkatasubramanian and Johnstone 1989). We have as yet no precise molecular description of how a germinant molecule initiates the series of physical, chemical and morphological changes which result in the breakage of dormancy. In general, theories have invoked either allosteric or metabolic roles for germinants (as in Halvorsen *et al.* 1966 and Prasad *et al.* 1972). Sensitive experiments designed to detect any metabolism in the germinating spore population suggested that commitment to germinate precedes any significant metabolism (Scott and Ellar 1978a,b).

Germination is insensitive to inhibitors of RNA or protein synthesis, and thus involves proteins already present in the mature spore. It is presumed that the germinant interacts with a specific site in the spore, which we may call a germination receptor; the assumption is that this generates some sort of allosteric alteration in the structure and properties of the receptor protein. If the receptor is located in a membrane, as discussed later, consequent changes in the membrane might alter its permeability properties, leading to a redistribution of ions and water in the spore and to activation of specific degradative processes (Keynan 1978). An alternative postulate is that the interaction of germinant with receptor leads to the activation by specific proteolysis of a cortex lytic enzyme (Foster and Johnstone 1988). These models need not be mutually exclusive. Models of the germination process are discussed at more length in a separate article (see Johnstone, this Symposium, pp. 17S-24S).

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physiological studies, and the relative difficulty of studying biochemical events during the rapid and relatively asynchronous germination of a spore population, suggested that a genetic approach might provide useful insights.

The logic of the genetic approach is that a mutant unable to germinate in the normal manner contains a mutation in a gene whose product is required for germination (either directly or indirectly). This approach defines a gene, and therefore a gene product, that is required (either directly or indirectly) for germination. The first steps, transfer of the mutation into an unmutagenized background then classification of the mutants and genetic mapping, have been invaluable in defining germination *ger* genes—and therefore the encoded Ger proteins.

Germination mutants were enriched in a population of spores by incubating them in a germinant, then challenging with heat or chloroform to kill any germinated spores. Because the population of wild-type spores germinates asynchronously, and may include some 'superdormant' spores, the procedure was repeated. Spores that had still not germinated, but that retained the ability to germinate under different conditions or after a longer lag, were recovered by plating on rich medium. Individual colonies were then purified, the putative mutants allowed to sporulate and the germination behaviour of washed spore suspensions tested (Trowsdale and Smith 1975). A plate test for scoring the germination phenotype of a colony, measuring the resumption of respiratory metabolism by the reduction of a tetrazolium salt, served as a quick reporter of germination phenotype, invaluable for genetic mapping and for the transfer of mutations between strains in genetic crosses.

3.1 Germination mutants

Spores of *B. subtilis* respond to at least two different types of germinative stimulus: they will germinate in alanine (ALA) or some analogues of this compound (valine or cycloleucine, for example), or in a combination of asparagine, which is not a germinant on its own, along with glucose, fructose and a potassium salt (AGFK: Wax and Freese 1968).

Varying the germinant included in the enrichment procedure generated different types of conditionally defective mutants; these were classified by phenotype and by map location, defining a number of *ger* genes (Moir *et al.* 1979). Each group of mutants that mapped to a different location on the genetic map, and therefore represented a different gene from the others, was given a separate genetic designation; they are discussed in more detail in Moir and Smith (1990), and are summarized in Fig. 1.

Like the *spo* loci, the *ger* loci are scattered around the chromosome of *B. subtilis*, rather than being clustered in

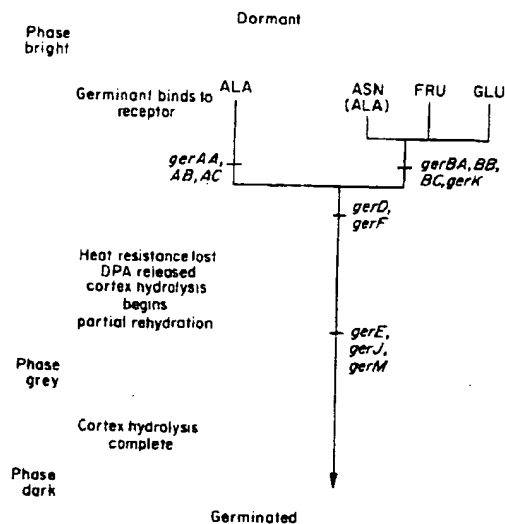


Fig. 1 A schematic representation of the mutational blocks in *Bacillus subtilis* spore germination mutants. DPA, dipicolinic acid

one place. The classical genetic approaches available could not distinguish whether a single locus contained a cluster of *ger* genes or only one: such an analysis had to await cloning of the *ger* loci.

Most of the mutants obtained were blocked before the loss of heat resistance (*gerA*, *B*, *D*, *F* and *K*); of these, *gerA* mutants are defective in alanine-stimulated germination (Sammons *et al.* 1981) but germinate normally in AGFK, whereas *gerB* mutants (Moir *et al.* 1979) and *gerK* mutants (Irie *et al.* 1982) fail to germinate in the latter mixture, but germinate normally in alanine. As the genes are required for the germination response to particular germinants, the simplest interpretation would be that the *gerA* and the *gerB/K* genes encode different germination receptors in the spore, responding to different stimuli. The suggestion that *gerA* encoded a receptor for alanine was reinforced by the isolation of *gerA38* and *gerA44* mutants, which have an increased concentration requirement for alanine and its analogues in ALA germination (Sammons *et al.* 1981). As some of the *gerA* mutations were responsible for temperature-sensitive germination, we can be confident that the GerA proteins act during germination, and are not regulators of the expression of the germination apparatus.

Two classes of mutant, those with *gerD* or *gerF* mutations, are affected in their response to germinants of both the ALA and AGFK type (Moir *et al.* 1979; Warburg *et al.* 1985; Irie *et al.* 1986). They were slow to germinate in ALA, and did not germinate at all in AGFK. This suggests that each of the gene products has a role that is essential for

AGFK germination, and that although both genes' products are involved in ALA germination, they are not essential for it to take place.

As these mutations are not germinant-specific, the gene products are not likely to be involved in germinant recognition; they have therefore been tentatively placed a little later in the series of germination events in Fig. 1; perhaps they are involved in transducing the initial germination stimulus?

Another group of mutants should never, logically, have been obtained by the enrichment procedures used; these are blocked at a later stage of germination, *after* the loss of heat resistance. The initial recognition of germinant by the spore is therefore still intact in these mutants. All three groups of mutants (*gerE*, *J* and *M*) have spore structural defects; these are examples of germination genes where the gene product influences spore germination without being directly involved. These germinate part-way, losing heat resistance, and starting but not completing the process of cortex hydrolysis (Moir 1981; Warburg and Moir 1981; Sammons *et al.* 1987).

Although not strictly germination genes, these represent interesting genes with a role in spore formation. The GerE protein is a DNA-binding protein that regulates expression of a number of spore coat genes (Errington 1993); the *gerE* mutant may lack a protein required for late stages of germination, either because its expression is dependent on GerE, or because the defective coats, which are permeable to lysozyme, allow the protein to leak from the spore.

Less is known about the function of GerM and GerJ proteins, although both types of mutant are known to be defective in sporulation. The altered heat-resistance properties and the late synthesis of spore-specific penicillin binding proteins in *gerJ* mutants (Warburg *et al.* 1986), and the multiple abnormalities in septum formation, cell division and cortex structure of *gerM* mutants, suggest that these, too, may be regulators of spore morphogenesis.

There are reports in the literature of metabolic mutants with defective germination, but these reports are of uncertain value. Unless the mutations are transferred into an unmutagenized background, it is always possible that the isolate carries two entirely separate mutations responsible for the two phenotypes. The genetic work reported in this section supports the hypothesis that metabolism of the germinant is not required. None of the mutants obtained are affected in metabolism. Germination mutants that lose the ability to germinate in alanine also lose the ability to germinate in non-metabolizable alanine analogues (Sammons *et al.* 1981), suggesting that their defect does not concern even a minor or alternative metabolic route. Glucose dehydrogenase null mutants germinate normally in germinant mixtures containing glucose (Irie, personal communication); this enzyme is therefore not concerned with the triggering

of the germination response. Currently, there is no proven case where a metabolic defect prevents spore germination.

3.2 Germination genes

Most of the *ger* genes listed in Fig. 1 have been cloned and sequenced, defining at least some of the proteins involved in the germination response. The discussion that follows concentrates on the cloned genes whose products are required for loss of heat resistance in response to germinant, i.e. *gerA*, *gerB*, *gerD* and *gerK* genes; the *gerF* gene has not yet been cloned.

Because *ger* genes do not confer any selectable characteristic on a host cell, cloning strategies have often been indirect. Some genes have been cloned in phage λ vectors. The *gerA* genes were obtained along with the adjacent *citG* gene, which was directly selectable in *E. coli* (Moir 1983). The *gerB* clone was obtained by chromosome walking from a nearby cloned locus (Corfe *et al.* 1994). The *gerK* gene was obtained by screening a large number of λ clones for the ability of their DNA to transform a *gerK* mutant to *ger*⁺ (Irie, personal communication).

An alternative general approach is based on Tn917 transposon technology (Youngman 1990); *ger* mutants are generated by an interruption of the gene by transposon Tn917. Modified versions of this transposon allow recovery from the chromosome of a section of DNA that includes one end of the transposon and the adjacent part of the interrupted *ger* gene. Once part of the gene has been cloned, it is possible to use this as a probe to screen a chromosomal gene library, and then recover the intact wild-type germination gene. This approach was used to clone the *gerD* gene (Yon *et al.* 1989).

Once cloned, information on the gene organization and regulation of expression of *ger* genes can be obtained by combinations of molecular and classical genetic techniques. The amino acid sequence of Ger proteins can be predicted, and strategies can be designed to overexpress the cloned gene products under the control of a foreign promoter.

The *gerA* locus contains three genes (*gerAA*, *AB* and *AC*), arranged in an operon (Feavers *et al.* 1985; Zuberi *et al.* 1985; Zuberi *et al.* 1987). The collection of known *gerA* mutants included mutations in each gene, indicating that all the GerA protein products are required for ALA germination. The *gerA38* and *A44* mutations that require higher concentrations of alanine for germination are both located in the middle gene, *gerAB*, suggesting that the GerAB protein is likely to bind alanine (Zuberi *et al.* 1985).

The regulation of expression of the *gerA* operon has been studied, by *lac* fusion analysis, promoter mapping and *in vitro* transcription (Feavers *et al.* 1990). As might have been predicted, the genes are subject to developmental control; they are not expressed in vegetative growth, but

3. THE GENETIC APPROACH

Biochemical approaches have not pin-pointed any specific protein that is yet proven to be necessary for germination. The plethora of models for germination derived from are switched on during sporulation in the forespore compartment, in response to the activity of the forespore-specific sigmaG-containing RNA polymerase. The level of expression of *gerA* genes appears to be very low, as judged by the very low level of expression of β -galactosidase from fusions to the *gerA* promoter (300-fold lower than expression from the fumarase (*citC*) promoter, for example), suggesting that the spore contains only low quantities of the GerA proteins.

The *gerB* locus contains three genes, *gerBA*, *BB* and *BC*, encoding three homologues of the GerA proteins, organized in the same order as the three genes of the *gerA* operon (Corfe *et al.* 1994; Fig 2). These too are dependent on the forespore-specific sigmaG-containing RNA polymerase for expression (Corfe and Moir, unpublished); the homology thus extends to their regulation. One difference is the level of expression: the *gerB* genes are expressed at an even lower level, about one-tenth of that of the *gerA* operon.

The *gerK* locus is at an earlier stage of analysis, but is already known to encode at least a homologue of GerBC and GerAC (Irie, personal communication; Fig. 2).

The *gerD* locus, in contrast, contains a single gene; it is expressed at a higher level (about 15-fold higher than *gerA*), but still under the control of sigmaG, in the forespore compartment of the sporulating cell (Kemp *et al.* 1991).

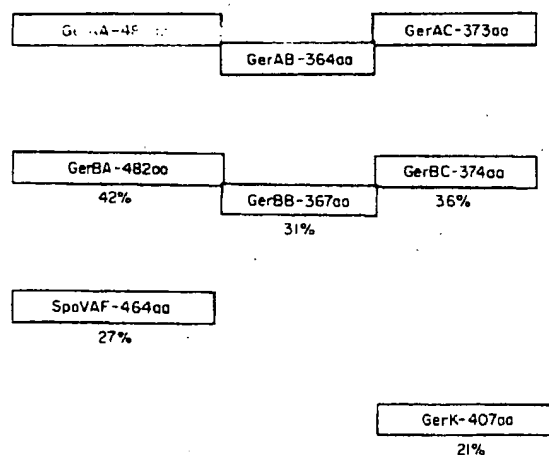


Fig. 2 GerA proteins and their homologues. Related proteins are aligned vertically. The percentage identity of amino acid residues with the corresponding GerA sequence is shown below the homologue

All the germination-specific *ger* genes so far analysed in detail (*gerA*, *B* and *D*) are expressed only during spore formation. The genes are all expressed in the forespore, which goes on to form the cellular compartment of the mature spore. As described in more detail below, the proteins encoded are predicted to be either membrane-associated or have N-terminal signal sequences, suggesting that they are variously located in, or transported across, the forespore membrane.

3.3 Germination proteins—their predicted properties

The 480 amino acid GerAA protein predicted from the DNA sequence would be organized in at least two, and probably three, distinct domains (Feavers *et al.* 1985; Fig. 3). Of predicted molecular weight 53 506 Da, it possesses a large hydrophilic (and therefore potentially cytoplasmic) N-terminal domain, followed by a membrane-associated domain of around 200 amino acids composed of five hydrophobic, potentially membrane-spanning helices, interspersed with charged regions. At the C terminus, there is another hydrophilic domain, of 50 amino acids—this is likely to be located on the opposite side of the membrane to the N-terminal one, if our prediction of five membrane-spanning helices (Fig. 3) is correct. The GerAB protein (41 257 Da) has the hydrophobicity profile characteristic of an integral membrane protein, with ten likely membrane-spanning helices (Zuberi *et al.* 1987). The GerAC protein (42 363 Da) is, in contrast, hydrophilic throughout, with the notable exception of a pre-lipoprotein signal sequence at the N-terminus (Fig. 3). The signal sequence suggests that this protein is transferred across the forespore membrane, and is predicted to be attached to the membrane via a cysteine linkage (Zuberi *et al.* 1987).

Comparisons of these proteins with protein sequence databases have not identified similarities with other types of protein, with the possible exception of the GerAB protein. Apart from GerBB, of course, the proteins that score highest against GerAB in FASTA alignment searches are an *Escherichia coli* tryptophan transport protein, TnaB (Sarsero *et al.* 1991), sharing 24% identity over 345 amino acids, a *Pseudomonas* arginine/ornithine transporter ArcD (Luthi *et al.* 1990) and Gram-positive tetracycline resistance proteins (Noguchi *et al.* 1986). There may be a distant evolutionary relationship between GerAB and the extended family of single component membrane transport proteins (Griffith *et al.* 1992).

The importance of the GerA group of proteins to germination has been underlined by the discovery that the GerBA, BB and BC proteins are 42%, 31% and 35% identical to their respective GerA homologues, suggesting that a basic mechanism has been conserved in receptors responding to different germinants, and that these receptors

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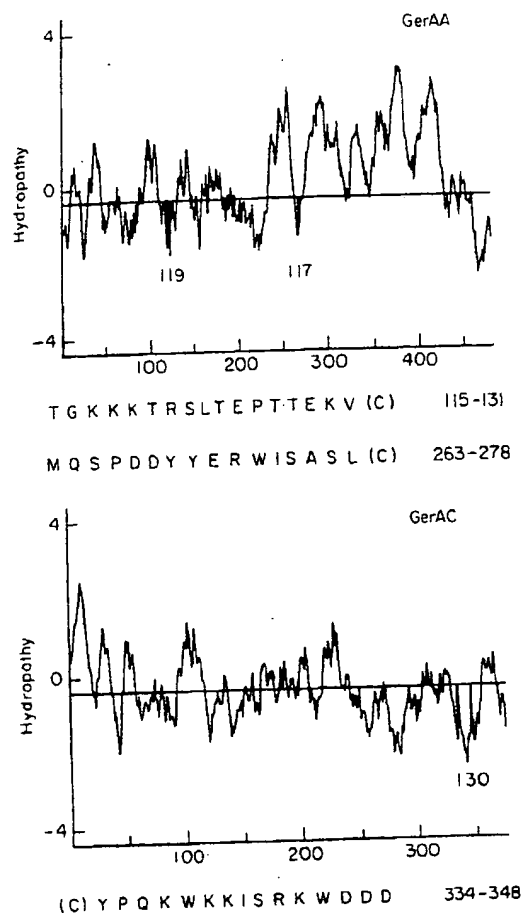


Fig. 3 The hydrophobicity profiles of GerAA and GerAC proteins. Also indicated are the synthetic peptides used for immunological studies. Peptides 119 and 117 were derived from hydrophilic sequences in GerAA, and peptide 130 from GerAC

have evolved by gene duplication and subsequent divergence. Figure 4 shows the alignment between GerAA and GerBA primary sequences. Most striking is the higher local homology in the GerAA/BA proteins in the 200 amino acid hydrophobic domain—especially the hydrophobic region of residues 346–391, where 36 out of 46 residues are identical. This degree of conservation, in proteins that otherwise have diverged considerably, suggests that these helices have a crucial functional importance, rather than being merely membrane anchors.

There are two more known members of this family (Fig. 2). The predicted GerK protein is a more distant homologue of GerAC and BC proteins (Irie, personal communication). In addition, the sixth protein encoded by the spoVA operon, SpoVAF, is a GerAA homologue, although there is no ALA or AGFK germination defect

GerAA	1	MEQTEFKEYIHONLALVLPKLNODLVKNKMLANG.LVFYLYFSNT	49
GerBA	1	...NQIDSDLQNNLDLTKTLGGQNDMMYPTFAFGDSRQKACLLYIDGLT	47
	50	DENKVSIAIKTLIKDEETL...TLGQVKRLDOLDARPVETAKTIESI	95
	48	ENHMLAQVVISPLQKEALAHKESIEDLSAFPFGPHSVVSTHKEIBOLV	97
	96	LMGCAVPIINGLDKAVILTGTGKKTRSLTEPTTEKVVRGPKVAFVEDIDT	145
	98	PSGQATLLADGYRGGLAPDKSVATRSLEPSSSEVVRGPKIGFIEKIRT	147
	146	NLALIRQRTSHPKLITKKNIGENKLPAAIMYIECKAKKSVIKVKARL	195
	148	NTALLRETSDDPNLVIKETLGRKTKKIAVAVIQIADPYVVKVKRL	197
	196	KNIQLEIDQSGTLEELIEDNKYSPPPTQINTERPKVSSALFNGRVAIL	245
	198	KSVNIDNLPESGTLLEQLIEDEPTSTPTILSTERPDRVSSLEGRYSIL	247
	246	VDSSPPVLLVVSIGLIMQSPDDYERWISASLIRSLRPASIFITLPLSS	295
	248	VDGTPPALIVTATVPEFHSPPDYSSQRTIPMSLVRLIRYSILITVTPG	297
	296	IYIALVSPHOGILLPTALAVTISANRENVPPPIFEALLMEVTIELLRAG	345
	298	LYISLVSPHOGILLPTALAVTISANRENVPPPIFEALLMEVTIELLRAG	347
	346	LPNPLGQTIGLVGGVIGQAAVEANLVSSILVIVVSVALASFTVPOY	393
	348	LRLPKPIGQTIGLVGGVIGQAAVEANLVSSILVIVVSVALASFTVPOY	397
	394	GMGLSPVRLRFISMPSAAIILGLYIILPMLVYVYTHLRTQTSFGSPSPN	443
	398	AYNPLRIIRICVNTISATAGNVEVTVYLVYVYTHLRTQTSFGSPSPN	447
	444	GFPS...LKNITDSDIIRLPKRNKPKVEVMNPKETDSTET	480
	448	AQPGQDLKDTVIRIPIPTNFKRPRTR.NQPDNIROR...482	

Fig. 4 A comparison of GerAA and GerBA proteins. The GAP program of the UWGCG sequence analysis package was used to generate the alignment. Identical residues are indicated by a vertical bar. The five likely membrane-spanning regions have been underlined and indicated by Roman numerals

associated with the inactivation of this gene (Kemp, unpublished).

The GerD protein (21 117 Da) is probably not receptor related, and has no homology with the other Ger proteins, or with any other protein in the database. It is hydrophilic, but does have an N-terminal signal sequence, suggesting that it is transferred from the forespore across the inner membrane or released into the peptidoglycan layer.

4. BACK TO BIOCHEMISTRY: EVIDENCE FOR LOCALIZATION

The proof of these sequence-derived predictions requires evidence that the genes do actually encode proteins of the expected size, and that these proteins are present in spores. This has been demonstrated for the GerA proteins. The *gerA* genes were cloned behind a regulatable, highly-expressed, *E. coli* promoter and expressed in an *in vitro* transcription/translation system; the labelled products from individual ger genes were assigned by expressing versions of *gerA* deleted for individual genes (Kemp et al. submitted), and they correspond approximately to the sizes predicted from the DNA sequence.

GerAA-derived and GerAC-derived synthetic peptides, chosen from the predicted amino acid sequence (Fig. 4), were conjugated to a carrier protein (ovalbumin) through

added cysteine residues. These antisera were used to probe Western blots of SDS-PAGE separated proteins from spore fractions. They identified a protein band, of the same size as *in vitro* expressed GerAA protein, in the spore membrane fraction (Fig. 5). This pattern was obtained with both of the two available anti-peptide probes. In contrast, the GerAC protein was found predominantly in the integument (coats and cortex) fraction (Kemp *et al.* submitted). The prediction had been that GerAC would be a lipoprotein attached to the outer surface of the forespore membrane; either this association is temporary, or not stable to the isolation procedure. More work will be needed to confirm this localization in the integument, using more gentle spore breakage procedures.

Interpretations have also to be qualified at the moment by the observation that there is some residual cross-reacting material of the same size as the GerA proteins in a GerA deletion strain: at the time that the peptide antibodies were

raised, the authors were not aware of the GerA homologues. Deletion strains are being used to clarify the situation.

The GerAB protein has not been studied in this way, but as a membrane protein synthesized in the forespore, it is unlikely that its location could be anywhere other than in the membrane bounding this compartment, i.e. the inner spore membrane, in the same location as GerAA. Whether the proteins are present in association is not known.

A fusion of part of the GerD protein to glutathione S-transferase has been overexpressed, affinity purified, and the GerD moiety cleaved from the fusion protein and used to raise polyclonal antisera in rabbits; the GerD protein is detected at the predicted size, in the integument fraction of the spore, and is absent in a *gerD* null mutant (Robinson, unpublished).

This work represents the first physical demonstration of germination proteins in the spore. Models of the mechanism of germination must take these data into account.

5. CONCLUSIONS

Bacillus subtilis contains genes that have evolved significantly from each other and that form families of proteins serving as the germination apparatus in the spore for different germinants. These are spore-specific proteins, that are not present in vegetative cells; there is no suggestion that they have a metabolic role in the spore.

We can now confirm that proteins that were predicted to form a germinant receptor in the spore are found in the integument layers (GerAC) and inner spore membrane (GerAA and AB). These proteins are the first recognized members of a wider family, and conserved sequences between different members of the family of homologues may point to particular regions of functional importance. It is not yet known whether their functions encompass functions other than spore germination—one member, SpoVAF, is not required for either of the known germination systems in *B. subtilis*.

The spore germination receptor and associated proteins represent a new class of sensory transducer, whose precise mode of action is unknown. It is not clear how individual germinants and groups of germinants act on particular receptors to initiate germination, or how the local interaction is transduced throughout the spore. Returning to the models of germination discussed earlier, it is possible that some of the Ger proteins are concerned with initiating ion fluxes: the membrane-associated proteins GerAA/BA and GerAB/BB are obvious candidates. Other proteins may be concerned with transducing the signal to the spore—could GerAC or GerD protein, for example, be a cortex lytic enzyme, or an activator of such hydrolytic activity? What would be responsible for their activation during germi-

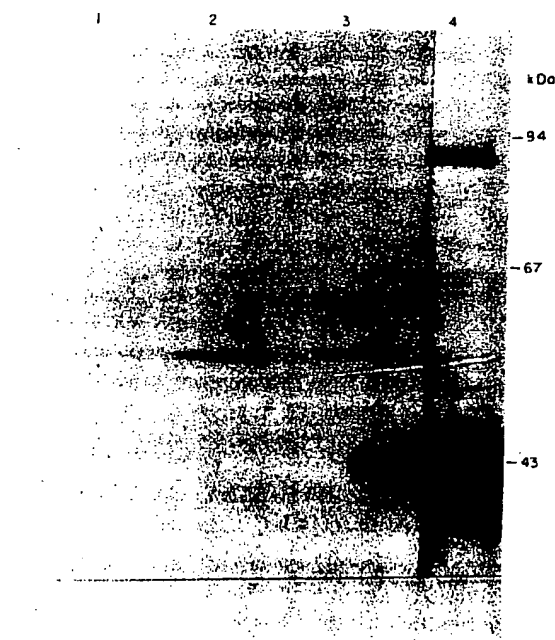


Fig. 5 The detection of GerAA protein in spore membranes. Spores were broken by homogenization with glass beads; the integument and membrane fractions were isolated in successive centrifugation steps. Proteins from each fraction were solubilized by boiling in SDS buffer and separated by SDS-PAGE. (100 μ g per lane). Lane 1, soluble fraction. Lane 2, membrane fraction. Lane 3, integument fraction. Lane 4, molecular weight standards (including ovalbumin). Antiserum raised against peptide 119 (GerAA) coupled to ovalbumin was used to detect cross-reacting proteins. A band of the size of GerAA was detected, primarily in the membrane fraction

nation? We now have a detailed molecular description of some of the components of the germination apparatus, but we do not yet have direct proof of the interaction with the germinant, nor do we know what happens when the germinant interacts with this receptor.

Although some progress has been made in identifying parts of the germination apparatus, it would be naïve to think that genetic analysis alone will be sufficient to generate a complete molecular explanation of the process. For example, procedures to isolate germination mutants would not allow detection of mutations in any gene whose protein product is a member of a functionally equivalent family, so that one of several proteins could substitute for the defective one. We are now entering a phase of research in which genetic and biochemical approaches and information need to be integrated; any generalizations drawn from a study of germination receptor proteins in *B. subtilis* need also to be tested in other bacilli. It would not be possible to adopt such a rigorous genetic analysis in other bacilli, but neither would it be necessary, as the tools for detecting homologous Ger proteins have been developed through the study of *B. subtilis*, and reverse genetics could be applied to test the relevance to germination of any cloned genes.

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Method 8.29 Determination of the retention capacity of Ultragel-IgG anti-le vansucrose (contributed by R. Chambert and M-F. Petit-Glatron, Institut Jacques Monod, CNRS-Université Paris, Paris, France) (see Section 8.3.6).

1. Mix various volumes of gel (0–250 μ l) with levansucrase (10 μ g) and 35 S-labelled levansucrase in 1 ml of 0.1 M potassium phosphate buffer, pH 7.2, containing 1% (w/v) BSA.
2. Incubate at room temperature for 3 h.
3. Filter the gel and wash extensively with PBS/BSA^a. Count the gel in scintillation liquid.
4. Determine the retention capacity of the gel from the curve of radioactive levansucrase coupled to the gel as a function of the IgG Ultragel volume. This is generally in the region of 100 μ g of levansucrase per ml gel suspension.

^a0.1 M sodium phosphate, pH 7.2; 0.15 M NaCl; 0.5% BSA (bovine serum albumin).

9

Sporulation, Germination and Outgrowth

Wayne L. Nicholson and Peter Setlow

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with contributions by

Alessandro Galizzi and Rachel Sammons

9.1 INTRODUCTION

Sporulation in *Bacillus* species is one of a series of responses to the cessation of exponential growth which is initiated after the end of cell growth and consists of a number of stages (stage 0, II, III, . . . through stage VII) which were originally classified by morphological features, and in more recent years by biochemical, genetic, and molecular genetic criteria (see Figure 9.1). Cells which are still growing are designated as stage 0, while stage I was initially defined as the stage at which two nucleoids of the vegetative cell condense to form a single axial chromatin filament. However, the stage I classification has generally been discarded, with the stage following stage 0 now referred to as stage II. During the stage 0–II transition sporulating cells synthesize a number of enzymes (mainly extracellular), including α -amylase, proteases, and nucleases, as well as a variety of extracellular antibiotics. Stage II is the point at which a division septum has been formed at one pole of the cell. This unusual cell division partitions the cell into quite unequal-sized compartments, each with its own chromosome. The larger compartment is termed the mother cell or sporangium, while the smaller compartment is termed the forespore or the prespore. As stage II proceeds, the forespore compartment is engulfed by the mother cell, resulting in stage III in which the forespore is free within the mother cell, and is surrounded by two distinct layers of cytoplasmic membrane of opposite polarity. During stage IV, peptidoglycan, both germ cell wall and cortex, is deposited between the forespore's two membranes, and in stage V spore coat proteins are deposited on the outside of the

tant spore can also survive for extremely long periods of time in the absence of exogenous nutrients. A large number of genes have been identified which are essential for sporulation but not cell growth. Some of these genes are expressed in growing cells, while others are expressed for only a defined period during sporulation, and often in only one of the two compartments of the sporulating cell.

9.2 INDUCTION OF SPORULATION

In general, sporulation is induced by limitation of the carbon, nitrogen, or (more rarely), phosphorus source in the environment. Nutrient limitation can occur naturally by allowing cells to exhaust nutrients in their growth medium either in solid or liquid media, or it can be imposed artificially by transferring the cells from a rich medium to a poor one. Additionally, sporulation can be induced under conditions of excess nutrients by chemical treatments which artificially lower the intracellular pool of GTP (see Section 9.2.2.d).

9.2.1 Solid media

Induction of sporulation on solid medium occurs by exhaustion of a nutrient in the medium. Sporulation can be monitored visually by the appearance of a characteristic brown pigmentation of sporulating colonies. Samples of the colonies can also be checked for sporulation microscopically. Spores will appear as phase-bright bodies by phase-contrast microscopy (or refractile bodies if viewed without phase-contrast optics), either free or within the mother cell. A variety of sporulation media have been formulated, but the solid medium most commonly used for sporulation of *Bacillus subtilis* is Schaeffer's sporulation agar (Schaeffer *et al.*, 1965). In general, sporulation on solid media is carried out at 37°C. However, sporulation mutants will rapidly lyse out on such media. Note that while solid media which do not support sporulation (such as LB, see Chapter 1) are used routinely to culture *B. subtilis*, care should be taken not to store important strains on these media, as the bacteria rapidly die upon exhaustion of the medium.

9.2.1.a Schaeffer's sporulation agar

The compositions of Schaeffer's sporulation agar, also known as Difco sporulation agar (DSM) and nutrient broth sporulation agar, are given in Appendix 1 (Schaeffer *et al.*, 1965). This medium can be used in liquid form by omission of agar.

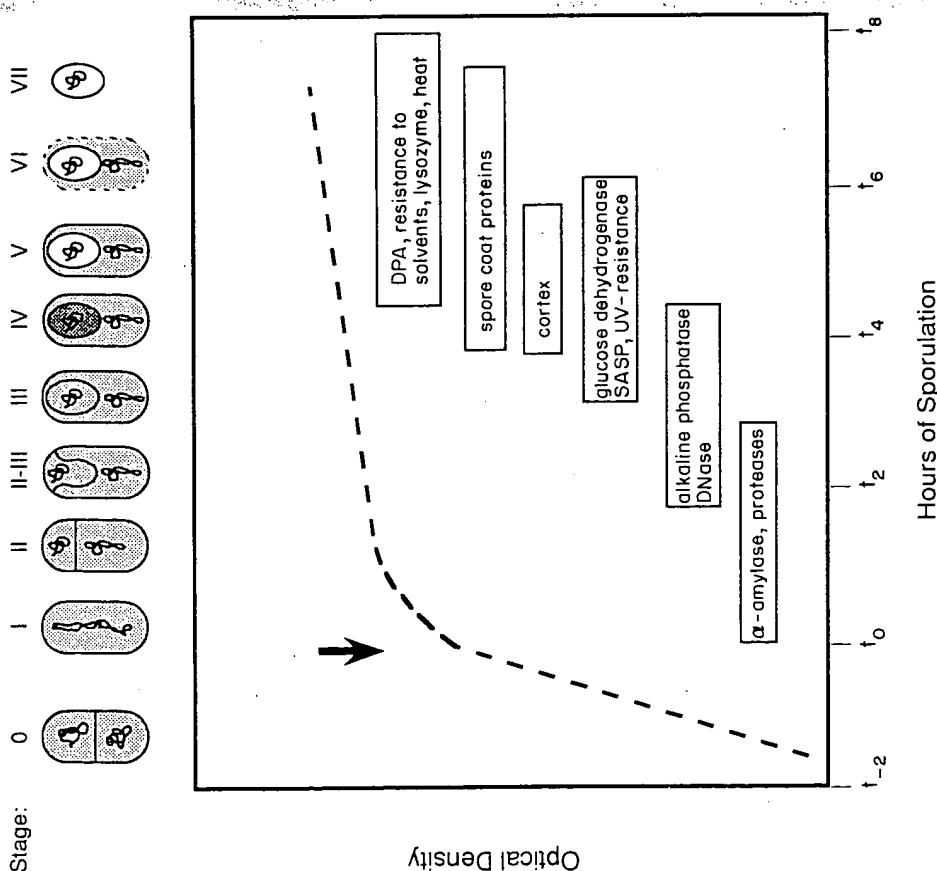


Figure 9.1 Idealized time course of sporulation in *Bacillus subtilis* at 37°C. Sporulation initiation in this system is defined as the end of exponential growth (arrow). Successive morphological stages of sporulation are schematically indicated above the graph at the approximate times of their occurrence. The timing and approximate duration of landmark biochemical and physiological events are indicated in the boxes within the graph. See text for details.

developing spore. During stage VI the spore 'matures' and develops full heat resistance, and in stage VII the mother cell lyses, releasing the mature spore. The released spore is now metabolically dormant, carrying out no detectable metabolism or macromolecular synthesis, and is much more resistant than growing cells to a variety of harsh treatments, including chemicals, heat, mechanical disruption and radiation. This dormant resis-

9.2.1.b 2× SG agar

This medium is a modification of Schaeffer's sporulation agar containing glucose, which can be used as a solid medium or, by omission of agar, as a liquid medium (Leighton & Doi, 1971). 2× SG is a richer medium, and gives higher cell densities than Schaeffer's medium. 2× SG agar consists of the following:

Per litre:	
Difco nutrient broth	16.0 g
KCl	2.0 g
MgSO ₄ ·7H ₂ O	0.5 g
agar	17.0 g

Adjust the pH to 7.0, autoclave, then add the following sterile component solutions to one litre of the cooled (55 °C) medium:

1 M Ca(NO ₃) ₂	1.0 ml
0.1 M MnCl ₂ ·4H ₂ O	1.0 ml
1 mM FeSO ₄	1.0 ml
50% (w/v) glucose	2.0 ml

9.2.2 Liquid media

Complex media such as Schaeffer's medium (Section 9.2.1.a) or 2× SG medium (Section 9.2.1.b) can also be used as liquid media (by omitting the agar, of course) for the induction of sporulation by exhaustion of growth substrates (Section 9.2.2.b).

Sporulation can also be induced by exhaustion of synthetic chemically defined media, and several media have been designed for this purpose (Donellan *et al.*, 1964; Freese *et al.*, 1978, 1979; Hageman *et al.*, 1984; Pascal *et al.*, 1971; Takahashi, 1979).

Sporulation can also be induced by resuspension of a growing culture in a poor medium (Section 9.2.2.c). Several resuspension media have been devised (Mandelstam & Wailes, 1968; Sterlini & Mandelstam, 1969; Ramaley & Burdon, 1970), but the most commonly used medium for this purpose in *B. subtilis* is Sterlini-Mandelstam sporulation medium (Sterlini & Mandelstam, 1969; see Section 9.2.2.c).

More recently it has been demonstrated that sporulation of *B. subtilis* can be induced in a defined medium which contains an excess of carbon, nitrogen, and phosphate (S7 medium; Freese *et al.*, 1979), by the addition of agents such as decoyinine which cause a decrease in the intracellular level of GTP (Section 9.2.2.d). S7 medium has more recently served as the base medium for the formulation of a chemically defined sporulation

medium (called CDSM) whose components have been adjusted to optimize growth and sporulation of *B. subtilis* 168 by the nutrient exhaustion method (Hageman *et al.*, 1984; see Section 9.2.2.a).

It is not certain precisely when the sporulation sequence of events is initiated in liquid medium. Time zero, the beginning of sporulation, is arbitrarily defined for experimental convenience as either the time at which cultures cease to grow exponentially in exhaustion medium, or the time of resuspension in sporulation medium if the resuspension method is used. Time zero is normally designated by the abbreviation t_0 , with t_1 , t_2 , etc. to denote the time (in hours) following t_0 , and t_{-1} , t_{-2} , etc. to denote the time preceding t_0 .

As with solid media, sporulation of *B. subtilis* in liquid culture is routinely carried out at 37 °C. As *B. subtilis* is a strict aerobe, for best spore yields cultures should be aerated vigorously, and the volume of liquid medium should ideally be kept at or below 15% of the total volume of the flask.

9.2.2.a CDSM medium

This medium is a modification of S7 medium (Section 9.2.2.d) which has been optimized as a single-stage growth and sporulation medium for *B. subtilis* (Hageman *et al.*, 1984) and can be used for induction of sporulation by the exhaustion method as described in Section 9.2.2.b.

Stock solutions

1.0 M	morpholinopropanesulfonic acid (MOPS)
0.05 M	KH ₂ PO ₄
1.0 M	(NH ₄) ₂ SO ₄
1.0 M	D-glucose
0.5 M	L-lactic acid
1.0 M	L-glutamic acid

Stock solutions are adjusted to pH 7.0 with KOH and autoclaved for storage, except for MOPS, which is filter-sterilized.

Trace metal mix (MT mix)

1 M HCl	2.0 ml
MgCl ₂ ·6H ₂ O	40.6 g
CaCl ₂ ·2H ₂ O	10.29 g
MnCl ₂ ·4H ₂ O	0.99 g
ZnCl ₂	13.6 mg
FeCl ₃ ·6H ₂ O	135 mg
thiamine-HCl	67.5 mg

Adjust volume to 1 l with distilled water. Add FeCl_3 last to prevent precipitation. Filter-sterilize, and store the MT mix in a dark bottle; discard if a precipitate forms.

Note that the recipes for MT mix for CDSM and S7 media are identical. However, the MT mix described above is a 50 \times stock for CDSM medium and is a 100 \times stock for S7 medium.

CDSM medium

CDSM medium is prepared by adding the stock solutions (above) to sterile distilled water to give the following final concentrations:

40 mM	MOPS
4.0 mM	KH_2PO_4
9.5 mM	$(\text{NH}_4)_2\text{SO}_4$
5.0 mM	L-lactic acid
8.0 mM	L-glutamic acid
1 \times MT mix	(see note above)
20 mM	glucose
50 $\mu\text{g}/\text{ml}$	tryptophan, isoleucine and other auxotrophic requirements

9.2.2.b Induction of sporulation by the exhaustion method

Method 9.1 gives a procedure for inducing sporulation by the exhaustion method. Normally, Schaeffer's liquid sporulation medium (Section 9.2.1.a) is used. 2 \times SG liquid sporulation medium (Section 9.2.1.b) can be substituted for Schaeffer's medium in this procedure although this nutritionally richer medium may make the determination of t_0 more ambiguous. If required, CDSM medium (Section 9.2.2.a) may also be used in this procedure.

9.2.2.c Induction of sporulation by the resuspension method

Method 9.2 outlines the procedure for inducing sporulation by resuspension in Sterlini-Mandelstam medium (SM medium). For this procedure the following are required:

<i>CH I + II</i>	
casein hydrolysate (Oxoid L41)	10.0 g
L-glutamic acid	3.68 g
L-alanine	1.25 g
L-asparagine	1.39 g
KH_2PO_4	1.36 g

NH_4Cl	1.34 g
Na_2SO_4	0.11 g
NH_4NO_3	0.10 g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	1.0 mg

Dissolve the glutamic acid in distilled water by adjusting the pH to 7.0 with 10 M NaOH. Then add the other ingredients, adjust the final volume to 940 ml, dispense into 94-ml aliquots and autoclave (15 lb/in², 30 min). Do not replace casein hydrolysate with casamino acids.

CH III

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.98 g
10% (w/v) CaCl_2	4.00 ml

Add separately to distilled water and bring the volume to 1 l. Autoclave and dispense into appropriate aliquots (100 ml).

CH IV

Dissolve 1.1 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ in 100 ml of distilled water, dispense into 10-ml aliquots and autoclave.

CH V

This is L-tryptophan (2 mg/ml) dissolved in water and filter-sterilized.

Sporulation salts

The recipe for this is given in Table 2.3.

Solution C

This is 5% L-glutamate. (L-glutamic acid; pH 7.0 with 10 M NaOH). Autoclave.

Solution D

This is 0.1 M CaCl_2 . Autoclave.

Solution E

1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Autoclave.

Growth medium

Growth medium (100 ml) is prepared on the day of use from the following sterile components:

CH I + II	94.0 ml
CH III	5.0 ml
CH IV	0.2 ml
CH V	1.0 ml

Tryptophan (CH V) is incorporated into the growth medium because this amino acid is lost during acid hydrolysis of casein.

Resuspension medium

Resuspension medium (100 ml) is prepared on the day of use from the following sterile components:

Sporulation salts	90 ml
Solution C	4.0 ml
Solution D	1.0 ml
Solution E	4.0 ml

The appropriate growth requirements **must** be added to this minimal medium (see Table 2.4).

The most difficult part of the procedure described in Method 9.2 is in obtaining exponential-phase growth after dilution in growth medium on the day of the experiment. Problems usually result from having allowed the cells to grow too far into stationary phase during growth overnight. This problem can be minimized by varying the conditions of the overnight culture. If a very fresh colony from an LB agar plate (Appendix 1) is used to inoculate the overnight culture at 37°C, by the following morning the cells can have an OD_{600} of >4.0 , which will cause a substantial lag phase even after dilution to an OD_{600} of 0.1. If a two- or three-day-old colony is used then the overnight culture will have a much lower cell density, so that they rapidly enter exponential phase and will reach an OD_{600} of about 0.5 within 1.5–2.0 h. Conversely, using a very old plate from which to inoculate the overnight culture may lead to very slow and poor growth in the overnight culture. Incubating the cells overnight at 30°C or even at room temperature may alleviate some of these problems. We use a roller-drum shaker in a 37°C warm room for incubating the overnight cultures and a rotary waterbath shaker for the sporulation cultures.

The growth and resuspension media must not be supplemented with antibiotics as this may interfere with growth and sporulation. When studying more than one strain it is important to remember that the cultures should be synchronized as far as possible. Subsequent cell sampling is more practical if all cultures are at the same stage in sporulation. This is facilitated by streaking out plate cultures at the same time and treating them in exactly the same fashion, i.e. diluting the overnight cultures to the same OD_{600} , resuspending at the same OD_{600} , etc.

We find that any residual growth medium in the centrifuge bottle does not interfere with subsequent sporulation, nor does returning the resuspended cells to the same culture flask. It is worthwhile following the OD_{600} as typically this will continue to increase up to about 1.0–1.5. After t_3 the OD_{600} is not representative, as the sporulating cells become optically refractile.

We find that resuspending cells at an OD_{600} of as low as 0.3–0.5 generates the most synchronous sporulation, and the highest percentage of sporulating cells. Expect to see long chains of cells, each containing a phase-bright prespore. Resuspending at a higher OD_{600} (0.7–1.0) will often produce large numbers of motile cells.

9.2.2.d Induction of sporulation by decoyinine addition

S7 medium is used for decoyinine induction of sporulation; a procedure to follow is given in Method 9.3. S7 medium contains a large excess of carbon, nitrogen, and phosphate. In the absence of decoyinine, sporulation is very poor (usually <10 spores/ml). If the final concentration of glucose is reduced from 2% to 0.4%, S7 medium can also be used as a defined sporulation medium (see Section 9.2.2.b) without the need for decoyinine. Decoyinine can be obtained from the Upjohn Company, Kalamazoo, MI. S7 medium is made up as follows:

S7 medium

Mix together and autoclave:

84 ml	distilled H ₂ O
0.5 ml	1 M potassium phosphate, pH 7.0 (prepared by mixing 1 M K ₂ HPO ₄ and 1 M KH ₂ PO ₄ to give a final pH of 7.0)
1.0 ml	1 M (NH ₄) ₂ SO ₄

Just before use, add:

10 ml	1 M morpholinopropanesulfonic acid (MOPS) (pH 7.0 with 10 M KOH)
2 ml	1 M L-glutamic acid (pH 7.0 with 10 M NaOH)
1 ml	MT mix (see Section 9.2.2.a)
4 ml	50% (w/v) glucose
	auxotrophic requirements (see Table 2.4).

9.3 SPORULATION-SPECIFIC MARKER EVENTS

Progress of *B. subtilis* cells through the sporulation sequence can be monitored by several morphological, biochemical, and physical criteria (see Figure 9.1). These have been reviewed extensively (Piggot & Coote, 1976; see Section 9.1), but will be briefly mentioned below.

Electron microscopy studies (Ryter, 1965) have resulted in the classification of the sporulation sequence into a series of distinct morphological stages designated by Roman numerals 0–VII (Piggot & Coote, 1976). Perhaps the easiest, although least precise, method for determining the

sporulation stage is simply by determining t_0 , the onset of stationary phase, and counting hours henceforth. During sporulation at 37 °C in wild-type strains, the morphological stage of sporulation is approximately the same as the hour of stationary phase (i.e. stage III occurs at about t_3 , etc.). Second rapid and somewhat more reliable method involves simple observation by phase-contrast microscopy, since some of the morphological stages defined by electron microscopy can also be distinguished under phase-contrast microscope. Early in sporulation (stage 0–II), cells will become strongly motile. Later, motility lessens and cells exhibit a slight arkening towards one pole of the cell. This dark area is due to the development of the spore protoplast (prespore or forespore) within the rod-shaped mother cell (stage III). The prespore becomes phase-dark and then progressively more phase-bright (stages IV, V, and VI), and finally the culture will consist almost exclusively of phase-bright spore bodies with no surrounding rod-shaped mother cells, due to their lysis (stage VII). A wide number of sporulation-specific events has been correlated with specific morphological stages (Figure 9.1). These events include synthesis of sporulation-stage-specific enzymes and secondary metabolites, and resistance to a variety of otherwise lethal chemical and physical treatments. The most widely used and convenient enzyme assays for monitoring sporulation stages are outlined below.

9.3.1 Assays of stage-0-specific enzymes

9.3.1.a Assay for extracellular α -amylase (Smith & Roe, 1949; Nicholson & Hambliss, 1985)

The assay described in Method 9.4 monitors disappearance of substrate, rather than appearance of product. It is best to assay each sample in triplicate and average the resultant values, due to natural variability in the assay. The assay is linear with respect to α -amylase up to a decrease of 0.6 A₆₂₀. The level of α -amylase in the medium is maximal at t_{3-4} .

9.3.1.b Assay for extracellular proteases (Dancer & Mandelstam, 1975)

Sporulating cells produce both a serine protease and a metalloprotease. The metalloprotease is inhibited by *o*-phenanthroline. Method 9.5 gives a procedure for assay of extracellular proteases. Omitting *o*-phenanthroline from this procedure will effectively measure total extracellular protease. Typically, protease activities peak at t_3 .

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9.3.2 Assay of a stage-II-specific enzyme

A procedure to measure the stage-II-specific enzyme alkaline phosphatase is given in Method 9.6. Alkaline phosphatase activity peaks in wild-type sporulating cells at t_5 (Glenn & Mandelstam, 1971; Errington & Mandelstam, 1983).

A second stage-II-specific enzyme which can be measured is extracellular deoxyribonuclease (DNase) (Akrigg, 1978; Akrigg & Mandelstam, 1978). However, assay of this enzyme requires production of [³H]thymidine-labeled DNA. Consequently, alkaline phosphatase is a much simpler stage-II-specific biochemical marker to assay.

9.3.3 Assay of a stage-III-specific enzyme

After stage III, forespores become increasingly resistant to disruption by lysozyme treatment. Therefore to assay stage III forespore-specific enzymes, such as glucose dehydrogenase (GDH), or β -galactosidase from *lacZ* fusions directed by $E\sigma^G$ -type promoters (Section 9.6), after stage III it may be necessary to first remove the spore coat layer by treatment with detergent and urea (Section 9.7.3) before proceeding with lysozyme treatment. Method 9.7 gives a procedure for assay of the stage-III-specific enzyme, GDH, in sporulating cells (Sadoff, 1966; Fujita *et al.*, 1977). Typically GDH activity is maximal at t_5 .

9.3.4 Assay of a stage-V-specific gene product

Method 9.8 gives the procedure for the determination of the stage-V-specific product, dipicolinic acid (DPA), in sporulating cells (Janssen *et al.*, 1958; Rotman & Fields, 1967). Typically, the DPA level is maximal at t_7 .

9.3.5 Determination of spore resistance properties

In addition to being resting, metabolically dormant forms, bacterial spores are much more resistant to a variety of environmental stresses than are their vegetative counterparts. Outlined in the following section are some of the ways in which *B. subtilis* spores are distinguished from vegetative cells on the basis of their differential resistances to such treatments as heat (Milhaud & Balassa, 1973), ultraviolet radiation (Mason & Setlow, 1986; Setlow, 1975; Setlow & Setlow, 1979), lysozyme (Jenkinson *et al.*, 1981), and organic solvents (Milhaud & Balassa, 1973).

9.3.5.a Ultraviolet (UV) light resistance

Resistance to UV light is acquired in stage III of sporulation, preceding acquisition of resistance to heat and organic solvents. UV resistance is due to the synthesis of small, acid-soluble spore proteins (SASP) and their association with the forespore DNA (Setlow, 1988). The assay for UV resistance generally involves constructing a UV survival curve for cells and spores at a given dose of UV light. Any short-wave UV lamp with a maximum output at 254 nm can be used. One such UV lamp is the model UVS-11 UV lamp (Ultraviolet Products, Inc., San Gabriel, CA). Lamps can be calibrated by measuring their output with an ergometer placed at various preset distances from the source (Setlow & Setlow, 1979). Method 9.9 details a procedure for the determination of UV resistance.

9.3.5.b Organic solvent resistance

Method 9.10 gives a general procedure to follow for assaying the resistance of spores to organic solvents.

9.3.5.c Heat resistance

A method for determining heat resistance is given in Method 9.11.

9.3.5.d Lysozyme resistance

Method 9.12 gives a procedure for determination of lysozyme resistance.

9.4 ISOLATION AND ANALYSIS OF SMALL, ACID-SOLUBLE SPORE PROTEINS (SASPs)

SASPs comprise approximately 7% of the protein of *Bacillus subtilis* spores, with three proteins, termed α , β , and γ , making up about 75% of the total SASP fraction (Setlow, 1988). These proteins are of two distinct types, an α/β type and a γ type. The α/β -type SASPs include SASP- α and - β , as well as many minor α/β type SASPs, each coded for by a unique gene. The α/β -type SASPs are closely related immunologically and have extremely similar amino acid sequences. In contrast, there is only a single γ -type SASP, encoded by a single gene. The γ -type SASP does not cross-react immunologically with antisera to α/β -type SASPs and has a very different amino acid sequence from them. All SASPs are synthesized within the developing forespore beginning approximately 3 h after the

onset of sporulation (i.e. at stage III). Transcription of the genes coding for SASPs (the *ssp* genes) is directed by RNA polymerase containing a forespore-specific sigma factor, σ^G (Sun *et al.*, 1989). SASPs are rapidly degraded to amino acids early in spore germination, thus supplying amino acids for much of the protein synthesis during germination and outgrowth. The α/β -type SASPs are also required for the resistance of the spore to ultraviolet light (Mason & Setlow, 1986; Section 9.3.5.a).

SASPs are relatively easy to isolate due to their solubility in dilute organic acids. However, they are extremely sensitive to proteolysis. Consequently, extraction from spores or sporulating cells at neutral pH often results in their degradation. SASPs are also quite small proteins (60–96 residues), and can readily pass through the pores in most commonly used dialysis tubing. Therefore, low-molecular-mass (< 5000 da) cut off dialysis tubing must be used for SASP isolation.

The most commonly used method for isolation of SASPs from *B. subtilis* prior to analysis is given in Method 9.13. This requires a dental amalgamator for dry rupture of cells or spores. The most commonly used dental amalgamator is the Wig-L-Bug Shaker, model 3110-B, produced by the Crescent Dental Manufacturing Co., and available through many scientific supply houses. The most efficient capsules to use with this instrument are the plastic screw-type (S.S. White Dental Products International, Philadelphia, PA). However, a 1/4-inch stainless-steel ball-bearing should be substituted for the pestle supplied with the capsules. Other procedures which have been tried for SASP extraction from sporulating cells include cryoimpaction and acid popping (see Method 9.14; Johnson & Tipper, 1981). However, cryoimpaction is more cumbersome than dry rupture, and acid popping only works well on mature spores which have been released from the sporangium. While the acid-popping procedure is simple and extracts all SASPs from spores, it also extracts other spore constituents including a number of minor SASPs (Johnson & Tipper, 1981; Setlow, 1978).

SASP analysis is routinely carried out by polyacrylamide gel electrophoresis at low pH, which separates all three major *B. subtilis* SASPs (Reisfeld *et al.*, 1963; Johnson & Tipper, 1981). Table 9.1 lists the solutions and reagents required for gel electrophoresis of SASPs at low pH. These gels should be run with the electrode polarity set appropriately for the fact that SASPs are positively charged in this gel system and will run towards the cathode. SASPs should be stacked in this gel system at 20 mA constant current, and run at 50 mA. Mobilities of the three major *B. subtilis* SASF relative to the ion front are: SASP- α , 0.25; SASP- β , 0.35; and SASP- γ , 0. (Johnson & Tipper, 1981; Mason & Setlow, 1986). SASPs can also be transferred from acrylamide gels to nitrocellulose paper for Western blot analysis using antisera to α/β - or γ -type SASPs (Goldrick & Setlow 1983).

Table 9.1 continued.

All solutions should be stored at 4 °C and are stable for > 3 months, except solution F, which should be made fresh weekly. The gel stain can be re-used, but should be discarded after staining five gels.

Preparation of gels

1. Allow solutions to warm to room temperature and prepare lower resolving gel as follows:

Solution	Relative amount
Lower resolving gel:	
A	1
C	2
F	4
H ₂ O	1

2. Pour into gel electrophoresis unit.
3. Layer water on top of this solution in the gel apparatus and allow to polymerize at room temperature for 1 h.
4. Remove the water and pour the upper gel, made as follows:

Solution	Relative amount
Upper stacking gel:	
B	1
D	2
E	4
H ₂ O	1

The total volume used for the upper gel should be 1/5 that used for the lower gel. The upper gel is polymerized for 30–60 min in strong sunlight; on a cloudy day a long-wavelength transilluminator can be used to promote polymerization. To run samples on a 15 × 17 × 0.15 cm slab gel, mix two parts dissolved SASP extract with one part diluent and run 2–50 µl of the extract. Gels are stained for 45 min with gentle agitation, and destained overnight.

9.5 EXTRACTION AND ANALYSIS OF SPORE COAT PROTEINS

Spore coat proteins are a group of proteins synthesized in the mother cell compartment of the sporulating cell (beginning at approximately *t*_{3.5} of sporulation and continuing until *t*₇), which are deposited in various layers around the outside of the developing spore. At least seven distinct spore coat proteins have been identified and six spore coat protein genes (*cot* genes) have been cloned from *B. subtilis* (Jenkinson *et al.*, 1981; Donovan *et al.*, 1987; Zheng *et al.*, 1988; Aronson *et al.*, 1989). Within the period of coat protein synthesis, different *cot* genes exhibit different temporal patterns of gene expression (Jenkinson *et al.*, 1981; Zheng *et al.*, 1988). The function of

Table 9.1 Reagents and solutions for gel electrophoresis of SASPs at low pH.

Solutions needed:

A. 1 M KOH	24.0 ml
glacial acetic acid	11.2 ml
tetramethylethylenediamine (TEMED)	2.3 ml
H ₂ O	62.5 ml
B. 1 M KOH	24.0 ml
glacial acetic acid	1.44 ml
TEMED	0.23 ml
H ₂ O	74.33 ml
C. acrylamide	28 g
bis-acrylamide	0.74 g
H ₂ O	to 100 ml
(filter solution C through Whatman #1 paper)	
D. acrylamide	10.0 g
bis-acrylamide	2.5 g
H ₂ O	to 100 ml
(filter solution D through Whatman #1 paper)	
E. riboflavin	0.4 g
H ₂ O	100 ml
F. ammonium persulfate	0.14 g
H ₂ O	100 ml

Running buffer (8×)

β-alanine	31.2 g
glacial acetic acid	8.0 ml
H ₂ O	to 1 litre
(dilute 1/8 before use)	

Sample diluent

glycerol	1.0 ml
solution B	2.0 ml
0.25% methyl green	0.25 ml

Gel stain

methanol	50 ml
glacial acetic acid	10 ml
H ₂ O	50 ml
Coomassie Brilliant Blue R	0.275 g

Destaining solution

methanol	75 ml
glacial acetic acid	50 ml
H ₂ O	875 ml

ne coat protein gene products is not known, although the product of *e* (*cotA*) is responsible for the dark-brown pigmentation associated with mature spores, while the product of the *cotE* gene may be a morphogenetic protein required for the appropriate deposition of other coat proteins (onovan *et al.*, 1987; Zheng *et al.*, 1988).

Several spore coat proteins exhibit rather abnormal amino acid compositions, with high levels of amino acids such as cysteine, histidine, proline or

Table 9.2 Reagents and solutions for SDS-PAGE analysis of spore coat proteins.

Solutions	
A. acrylamide bis-acrylamide dH ₂ O (filter solution A through Whatman #1 paper)	30.0 g 0.5 g to 100 ml
B. acrylamide bis-acrylamide dH ₂ O (filter solution B through Whatman #1 paper)	10.0 g 0.76 g to 100 ml
C. 1.5 M Tris.Cl, pH 6.8	100 ml
D. 0.5 M Tris.Cl, pH 6.8	0.4 g 100 ml
E. ammonium persulfate dH ₂ O	0.4 g 100 mg to 1.0 ml
F. tetramethylethylenediamine (TEMED)	
Running buffer (8×)	
Tris base	24.0 g
Glycine	115.2 g
H ₂ O (dilute running buffer to 1× and add SDS to 0.1% final concentration just prior to use)	to 1 litre (pH 8.3)
Gel stain	
2-propanol	25 ml
glacial acetic acid	10 ml
dH ₂ O	56 ml
Coomassie Brilliant Blue R	0.2 g
Gel destain	
2-propanol	100 ml
methanol	50 ml
glacial acetic acid	100 ml
dH ₂ O	750 ml
Gel storage	
ethanol	5 ml
glacial acetic acid	10 ml
dH ₂ O	85 ml

Table 9.2 continued.

Bromophenol blue
Solutions A and B should be kept at 4 °C in the dark. Solutions A through F should be stored refrigerated and are good for at least 3 months, except for solution E which should be prepared fresh daily. Gel stain should be used to stain only four gels and then discarded.

Gel formulation

	Solution	Volume (for 32 ml gel)
Lower resolving gel:	A	16 ml
	C	4 ml
	E	160 µl
	F	16 µl
Upper stacking gel:	H ₂ O	11.82 ml
	B	5 ml
	D	2.5 ml
	E	100 µl
	F	10 µl
	H ₂ O	2.4 ml

The total volume used for the upper gel should be 1/5 that used for the lower gel.

tyrosine (Aronson *et al.*, 1989; Donovan *et al.*, 1987). All spore coat proteins are relatively insoluble at neutral pH and require either detergents or high pH for solubilization. The proteins can be extracted either from intact spores (Methods 9.16 and 9.17) or from a spore coat fraction isolated from broken spores (Method 9.15). The proteins are routinely analyzed by SDS-PAGE (Table 9.2).

9.5.1 Isolation of spore coat proteins

Method 9.15 gives a procedure to isolate the spore coat fraction from disrupted spores, and Methods 9.16 and 9.17 give procedures for detergent extraction or alkali extraction of spore coat proteins respectively.

9.5.1.a Potential problems

The two different extraction methods extract a different group of spore coat proteins. Method 9.16 gives the highest yield of spore coat proteins, while the alkali extraction (Method 9.17) removes very little of the 34- and 38-kDa spore coat proteins. Consequently, Method 9.16 is generally the one of choice for completeness of extraction. While the major coat proteins isolated from the spore coat fraction and intact spores by alkaline-SDS

appear identical by SDS-PAGE analysis, some proteins **not** from the spore coat are often found in extracts of intact spores. This may be due to lysis of a small percentage of spores during detergent extraction. While it is possible that some alteration of individual spore coat proteins takes place during isolation of the spore coat fraction, this appears to be a minor problem.

9.5.2 SDS-PAGE of spore coat proteins

All major spore coat proteins can be resolved for visual analysis by electrophoresis through 1.5 mm-thick-SDS-PAGE slab gels. Table 9.2 lists the solutions and reagents required for SDS-PAGE analysis. Briefly, pour the lower gel, layer water over the top of the gel solution and allow to polymerize. Then pour off the water, insert a comb, add the upper gel solution, layer water over the top, and let polymerize.

To run the gel, make the spore coat protein samples 0.002% in bromophenol blue and incubate for 5 min at 100 °C. Apply the samples to the gel and run the gel at 25 mA until the dye is 0.5–1.0 cm from the bottom. Gels are stained for 14 h in solution H, destained in solution I until the background is colorless, and stored in solution J. For better resolution of some individual coat proteins, the gel concentration in the resolving gel can be increased to 18% (to better resolve the 11- and 12-kDa proteins) or decreased to 12% (to better resolve the 59- and 65-kDa proteins).

9.6 SEQUENTIAL GENE EXPRESSION

One of the reasons for the interest in the process of sporulation in *Bacillus* species is that it is a simple differentiation process. One feature of this differentiation is that during sporulation there is a defined temporal pattern of gene expression. Some genes are expressed only during vegetative growth, and are turned off during sporulation itself. Other genes are turned on only during sporulation, and in this group of genes there are many different temporal patterns of expression. However, a common finding with sporulation-specific genes is that they are expressed for only a defined 1–3 h period in sporulation (Losick & Kroos, 1989).

Exact knowledge of the time of expression of a sporulation specific gene is important because: (i) it provides suggestive information about how the gene is regulated (i.e. which form of RNA polymerase **might** transcribe the gene—see Chapter 6); and (ii) it may well indicate if expression of the gene is cell-compartment-specific—mother cell or forespore—since genes expressed after t_2 – t_3 may be expressed in only one of these compartments (see Section 9.7).

Clearly the best way to determine the time period of a gene's expression is to directly measure the level of the gene's product throughout growth and sporulation. While this has been accomplished for some sporulation-specific genes, for many such genes there is no assay for the gene product. However, if a gene has been cloned, its expression can readily be monitored by fusing the gene's regulatory elements to the gene for *E. coli* β -galactosidase (*lacZ*) using one of a variety of cloning vectors (Chapter 5), and then monitoring β -galactosidase levels throughout growth and sporulation as described below (Sections 9.6.1 and 9.6.2). This approach has now been used successfully to determine the temporal (and spatial) pattern of expression of a large number of sporulation-specific genes and, where examined, it has given essentially identical results to those obtained by direct assay of the corresponding gene's product (Mason *et al.*, 1988; Setlow, 1989).

9.6.1 General procedure

1. A strain carrying a *lacZ* fusion to a gene of interest is grown and sporulated by nutrient exhaustion (Method 9.1) or the resuspension technique (Method 9.2). Samples (1.0 ml) of culture are harvested by centrifugation in a microfuge at hourly intervals, the supernatant fluid is removed and the pellet is stored frozen. It is often desirable to take multiple samples at each time point if assays in addition to β -galactosidase are to be carried out. It may also be essential to obtain samples from an isogenic strain which lacks the *lacZ* fusion, grown in parallel with the strain carrying the *lacZ* fusion (see below and Section 9.6.3).
2. Generally, sampling is begun during midlog-phase growth and continued until approximately t_8 , with a final sample being taken at about t_{24} .
3. After all sampling is complete, samples are assayed for β -galactosidase using either ONPG (o-nitrophenyl- β -D-galactoside) or MUG (4-methylumbelliferyl- β -D-galactopyranoside) as a substrate, after opening the cells by lysozyme or toluene treatment as described in Method 9.18. The choice of ONPG or MUG as a substrate is dictated by the level of β -galactosidase activity produced from the *lacZ* fusion. In general, levels of activity below 5–10 Miller units (Miller, 1972) with ONPG are reassayed with MUG. However, for fusions which give low levels of β -galactosidase activity it is essential to also assay the β -galactosidase levels in the parental strain lacking the *lacZ* fusion.
4. With an approximate temporal sequence of gene expression obtained as described above, it is often tempting to directly assign a sporulation stage for the gene's expression based on the optical density profile of

the culture (or the time after resuspension). However, a more definitive temporal framework can be assigned if some of the duplicate samples are assayed for other known sporulation-specific gene products such as alkaline phosphatase, glucose dehydrogenase, or dipicolinic acid (Section 9.3).

9.6.2 Assay of β -galactosidase activity in spores

Method 9.18 gives two representative assays for β -galactosidase activity, using as the substrate either ONPG (Miller, 1972; Mason *et al.*, 1988) or MUG (Youngman *et al.*, 1985; Zuberi *et al.*, 1987).

Extraction of cells with lysozyme measures enzyme activity in both mother cell and forespore until the forespore becomes lysozyme resistant (Section 9.7.3). However, toluene treatment allows assay only of enzyme in the mother cell compartment. In some cases, lysozyme extracts may not pellet well. However, centrifugation for up to 10 min, and removal of only the top layer of the supernatant fluid, generally allows accurate OD₄₂₀ readings. If this is a serious problem with a particular strain, DNase can be included with the lysozyme as in the MUG procedure.

9.6.3 Potential problems

One problem with the use of *lacZ* fusions to determine the temporal pattern of a gene's expression during sporulation was alluded to above, namely that *Bacillus* species have their own β -galactosidase. Consequently, there is a background of enzyme activity in strains which do not carry *lacZ* fusions. More significantly, this activity rises during sporulation, reaching values of 1–3 Miller units. Therefore, assays on control cultures are essential in analyses of low-level-activity *lacZ* fusions.

A second potential problem concerns the finding that some *lacZ* fusion products, particularly those expressed in the mother cell alone, or only between *t*₀ and *t*₂, are relatively unstable. This could lead to erroneous conclusions about the time course, or compartment specificity, of gene expression. For example, levels of β -galactosidase might suggest that a gene's expression halts at *t*₂ when in reality β -galactosidase is being simultaneously synthesized and degraded. Similarly, assays of β -galactosidase might indicate that a gene is expressed predominantly in the forespore, when in reality the gene is expressed equally in both mother cell and forespore compartments, but the fusion protein is preferentially degraded in the mother cell. It is possible that use of different *lacZ* fusion products (such as transcriptional fusions) might alleviate the problem of instability of β -galactosidase activity in the mother cell during sporulation.

However, insufficient data are available at this time to suggest a definitive remedy for this problem.

A third problem sometimes encountered in these experiments is that the expression of a *lacZ* fusion may take place only in the forespore. Consequently, the β -galactosidase activity derived from this *lacZ* fusion will become increasingly refractory to direct lysozyme extraction as the developing forespore becomes lysozyme resistant. The result will be a drastic fall in β -galactosidase activity, making precise assignment of the timing of the gene's expression difficult. However, this problem can readily be overcome if information on the compartment specificity of this gene's expression is obtained, as described in Section 9.7.

9.7 COMPARTMENTALIZATION

One of the key morphological events early in sporulation is an unequal cell division resulting in the larger mother cell compartment and the smaller forespore compartment. While some genes are undoubtedly expressed at similar levels in both compartments, much interest has focused on genes which are expressed after this unequal cell division, and in only one of these two cell compartments. An important step in characterization of such genes is the determination of the site of their expression—mother cell or forespore. Several methods have been devised to allow this determination, including the use of genetic complementation (de Lencastre & Piggot, 1979), isolation of forespores (Fujita *et al.*, 1977; Singh *et al.*, 1977) (Methods 9.19 and 9.20), and differential extraction of mother cell and forespore compartments (Mason *et al.*, 1988; Method 9.21), as well as a variety of other approaches. The procedure described in Method 9.21 is extremely useful in analyzing distribution of β -galactosidase from a *lacZ* fusion, as the SDS-urea treatment inactivates all enzyme in the mother cells and the lysozyme-sensitive forespores, while not inactivating enzymes in the lysozyme-resistant forespores. It also allows assessment of the percentage of gene product made during sporulation which becomes incorporated into mature spores. In general, forespore-specific gene products appear in the lysozyme-resistant fraction in parallel with acquisition of dipicolinic acid. Consequently, these analyses are best performed by analyzing two cell samples, one by direct lysozyme treatment (Method 9.18), and one by lysozyme extraction after SDS-urea treatment as described here.

Note that a key piece of information needed before concerns about the location of expression of a sporulation-specific gene can be addressed is the time at which that gene is expressed. Genes expressed well after *t*₂ of sporulation may be expressed in only one cell compartment, while genes expressed at *t*₀–*t*₁ are not.

9.7.1 Determination of compartment-specific expression by transformation of a Spo^- strain

This procedure (de Lencastre & Piggot, 1979) involves transformation of a Spo^- strain, just prior to t_0 and completion of DNA replication, using wild-type DNA. The culture is allowed to complete sporulation and about 10^3 spores are individually scored as Spo^+ or Spo^- , usually by their colony pigmentation on sporulation agar. spo genes which give rise only to genetically Spo^+ spores after transformation are scored as forespore-specific genes, while spo genes which give rise to a significant percentage ($> 10\%$) of genetically Spo^- spores are either mother-cell-specific genes or are expressed prior to septation at t_2 , with continued forespore-specific expression not required for sporulation. The principle of the technique is that the wild-type transforming DNA taken up at t_0 can partition either into the mother cell or the forespore. However, only when the wild-type transforming DNA is captured in the forespore can a strain carrying a mutation in a necessary forespore-specific spo gene sporulate; the spores thus formed will be genetically Spo^+ . In contrast, strains which carry mutations in mother-cell-specific spo genes, or in spo genes expressed before septation, will sporulate if they take up the wild-type spo allele at t_0 , but a proportion of the resulting spores will be genetically Spo^- .

One of the main drawbacks to this procedure is that it can only readily be applied to genes in which mutations give a clear phenotype, i.e. Spo^+ or Spo^- . Since mutations in a number of forespore-specific genes give no clear phenotype, this has proven a poor method for the identification of forespore-specific genes. However, for genes essential for sporulation it can prove useful to determine in which compartment a gene's expression is required. One advantage of this procedure is that a gene's expression can conceivably be assigned to a compartment without any knowledge of the gene product, and without having cloned the gene!

9.7.2 Forespore isolation

9.7.2.a Forespore isolation using a French press

Method 9.19 gives a procedure for isolating forespores using a French pressure cell.

9.7.2.b Forespore isolation using sonication

Method 9.20 gives a procedure for isolating forespores using sonication.

9.7.2.c Potential problems

There are several potential problems with Methods 9.19 and 9.20 for forespore isolation. The first is that it is essential that cells are uniformly

suspended prior to lysozyme treatment, to ensure that all mother cells will become sensitive to mechanical disruption. It is often helpful to examine an aliquot of the lysozyme-digested suspension in a microscope to ensure that the great majority ($> 95\%$) of cells have become spheroplasts prior to French press or sonication treatment. A second problem with these procedures is that, while they work well for the isolation of forespores from cells at t_4 – t_6 of sporulation, yields of t_3 forespores are significantly lower, and prior to t_3 forespores are too fragile to survive and are not recovered. Consequently, isolated forespore populations tend to be enriched in older forespores relative to the starting cell population. The third problem with both of these procedures is that, while they do provide significant fractionation of mother cell constituents and forespores, this fractionation is not exact, and mother cell constituents are always contaminated with forespore constituents, and vice versa. Although with good preparations this cross-contamination may be only 5–10%, it can be a significant problem. Consequently, when assaying the mother cell and forespore extracts prepared by these procedures for the product of some uncharacterized gene, it is often useful to analyze a known mother-cell- or forespore-specific gene product at the same time. One forespore-specific gene product which is easy to assay is glucose dehydrogenase (Section 9.3.3).

9.7.3 Differential extraction of mother cell and forespore

While fractionation of sporulating cells into mother cell and forespore compartments can allow determination of the site of expression of a particular gene, this technique is difficult to carry out on multiple samples during sporulation. A different approach to answering the question of location of gene expression is to take advantage of the fact that the developing forespore becomes resistant to lysozyme treatment as coat proteins are deposited around the spore. Consequently, forespore-specific gene products produced at t_3 – t_4 will initially be extracted with lysozyme, but will become increasingly refractory to lysozyme extraction as sporulation proceeds (Mason *et al.*, 1988). However, if sporangia containing lysozyme-resistant prespores are first treated with SDS and urea to solubilize spore coat proteins, the forespore contents can then be readily extracted with lysozyme (Mason *et al.*, 1988). A procedure to render normally lysozyme-resistant forespores lysozyme sensitive is given in Method 9.21.

9.7.4 Other approaches to determine compartmentalization of gene expression

A number of other techniques have been used to determine the compartment-specific pattern of expression of a particular gene. One which can be useful for suspected forespore-specific genes is to isolate

spores at $\approx t_{24}$ and analyze the level of gene product extracted by lysozyme before and after spore germination (Errington & Mandelstam, 1986). Under these conditions forespore-specific gene products will only be extracted from germinated (i.e. lysozyme-sensitive) spores. Again it is important to assess the percentage of the gene product made in sporulation which is present in the mature spores.

An alternative approach for determining the compartment in which a particular gene is expressed is to determine the dependence of the expression of this gene on other previously characterized *spo* loci. Of particular utility in this analysis are mutations in forespore-specific (*sigG*) or mother-cell-specific (*spoIIID*, *sigK*) regulatory loci (Mason *et al.*, 1988; Losick & Kroos, 1989). However, while this type of analysis can give strong hints as to the site of a gene's expression, it is no substitute for its direct determination.

A third general type of approach for determining the site of a *spo* gene's expression is to use antisera against the wild-type gene product and carry out immuno-electron microscopy on sporulating cells at stage III–IV. While this has successfully localized the abundant forespore-specific gene products SASP- α , - β , and - γ (Francesconi *et al.*, 1988), it is a rather specialized technique and requires good antisera against a wild-type gene product. However, since antisera against β -galactosidase is readily available commercially, this may prove to be a very rigorous method for localizing the products of a *lacZ* fusion.

9.8 PURIFICATION OF SPORES

While analysis of many sporulation events, including those unique to the forespore compartment, can be carried out on sporulating cells, some analyses require cleaned free spores. This is particularly true for initial analyses of the distribution (mother cell or spore) of a newly discovered gene product, and for purification of spore-specific gene products such as coat proteins or SASPs. In many respects the most important determinant of the ease of spore purification from liquid cultures is the efficiency of sporulation. If this is very high, the great majority of mother cells and unsporulated cells in the population will lyse as sporulation proceeds, leaving only free spores and cell debris in the culture. In practice, this ideal situation is never observed in *B. subtilis*, but use of efficiently sporulating strains and good sporulation media gently facilitates spore purification. Strains which sporulate well are 168 (BGSC 1A1) and JH642 (BGSC 1A96). While good spore crops can be obtained 24 h after inoculation, it is often advisable to allow cultures to continue shaking for an additional 24 h to permit further cell lysis to take place. The loss of contaminating cells

can be conveniently followed by using a phase-contrast microscope. Alternatively, if only small amounts of cleaned spores are needed, sporulation can be carried out on solid medium until cell lysis is complete.

A number of procedures have been described for spore purification. The simplest (Section 9.8.1) involves extensive water washing over a period of 1–2 weeks, during which time autolysis allows removal of cells, sporulating cells, and debris. Other procedures (Section 9.8.2) (Jenkinson *et al.*, 1981) utilize lysozyme treatment followed by extensive washing with salt and SDS solutions. In some cases (Section 9.8.3) spores may be isolated from cells earlier in sporulation using a French press to disrupt the sporulating cells and release the free spore (Jenkinson *et al.*, 1981). Partially purified spore preparations can also be purified by centrifugation on Urografin (Renografin) density gradients (Section 9.8.4).

9.8.1 Spore purification with water washing

Centrifuge 48 h cultures (10 000g, 10 min) and wash three times with 1/4 volume of cold water on day 1. Generally the pellet will show several layers, with the bottom layer being mainly free spores, the next layer mainly sporulating cells with some free spores, and the top layer mainly cell debris with some cells and spores. Often the top layer is very loose, and will rapidly slide down a centrifuge bottle when inverted. However, retain all layers on the first day and shake the entire pellet fraction gently overnight at 4 °C in 1/5 the original culture volume of distilled water. On subsequent days centrifuge the suspension once (20 000g, 20 min) and resuspend in distilled water. Eventually (within 3–10 days) there is only a single layer over the free spores. This contaminating layer becomes very viscous and sticks together tightly when swirled in water or gently squirted with a water bottle. At this point the entire upper layer can be removed by gently squirting with a water bottle and decanting. The remaining pellet is almost exclusively free spores which stream away uniformly when sprayed with a water bottle. After several more days of water washing the spores are ready for further use.

9.8.2 Spore purification by lysozyme treatment and salt and detergent washes

Harvest cultures at t_{24} or later by centrifugation (10 000g, 10 min, 4 °C). Wash the pellets with 1/4 volume of 1 M KCl/0.5 M NaCl and incubate at 37 °C for 60 min in 1/4 volume of Tris.Cl (50 mM, pH 7.2) containing lysozyme at 50 μ g/ml. Clean the spores by alternate centrifugation (10 000g, 10 min) and washing with: (1) NaCl (1 M); (2) deionized water; (3) SDS (0.05%); (4) TEP buffer (50 mM Tris.Cl buffer, pH 7.2, containing 10 mM

EDTA and 2 mM phenylmethylsulfonyl fluoride); and (5) three washes with deionized water.

9.8.3 Release of mature spores from sporangia by using a French press

Harvest cultures in which spores are still retained within sporangia $\approx t_6-t_{10}$ by centrifugation (10 000g, 10 min) wash once with 1/4 volume of 1 M KCl and resuspend to a density of approx 5 mg dry weight per ml in TEP buffer (see Section 9.8.2). Pass the suspension twice through an ice-cold French pressure cell at 83 MPa to release spores from mother cells, and dilute 10-fold with cold TEP buffer. Harvest the spores by low-speed centrifugation (2000g, 5 min), and wash several times with TEP buffer by centrifugation at higher speed (10 000g, 10 min), with removal of any cell debris overlaying the spore pellet.

9.8.4 Spore purification on urografin gradients

The solution needed is Urografin (76%, w/v) which can be obtained from Squibb and is sold as Renografin in the USA. It is also sold as the sodium salt of metrizoic acid (75%, w/v, Sigma).

Pellet partially purified spore preparations from 100–200 ml of culture by centrifugation (10 000g, 10 min) and suspend the pellet in 2 ml of 20% Urografin. Layer this suspension gently over 10 ml or 20 ml of 50% Urografin in a 15-ml or 30-ml glass Corex centrifuge tube, and centrifuge the tube for 30 min at 11 000 rev/min in a Sorvall SS-34 rotor. The pellet contains only free spores, and should be washed three times with deionized water to remove residual Urografin. Often layers or bands will be seen in the Urografin—the uppermost is vegetative cells; the band 1/5th of the way down is cells with enclosed spores.

9.8.5 Potential problems

While all the procedures described above can give preparations containing > 98% free, phase-bright spores, they have different drawbacks. With water washing there are occasional spore crops which do not clean up well, generally because sporulation is not very efficient or because too high a percentage of spores have not been released from the sporangium at the time of harvesting. These crops can either be discarded, or lysozyme or French press treatment can be used. A second disadvantage of water washing is that some mother cell constituents may adsorb to the mature spores upon mother cell lysis, and are carried through with the free spores. Possibly, washing spores as described in Section 9.8.2 after lysozyme treatment would eliminate the problem. The procedure using lysozyme

treatment does not have the problem of mother cell component adsorption to the forespores, but with some spore crops lysozyme treatment is either not complete or can lead to significant spore germination. The procedure in Section 9.8.3 also suffers from the general incompleteness of sporangial rupture, and from the requirement for a French press. Purification of spores on Urografin gradients always gives clean spores, even with poorly sporulated cultures. However, Urografin is not inexpensive, and is costly to use to prepare very large spore crops. Nevertheless, for preparation of small amounts of highly purified spores from multiple samples, it is the method of choice.

9.9 STORAGE OF PURIFIED SPORES

All procedures for spore purification end with spores being suspended in cold, deionized water; this is suitable for short-term storage (3–5 days). However, for longer-term storage (> 1 week) of purified spores, other possibilities should be examined. Two general procedures have been used for long-term storage of purified spores, each with advantages and disadvantages.

9.9.1 Storage of lyophilized spores

Purified spores are centrifuged (10 000g, 10 min) and the resulting pellet is lyophilized. The dried spores are then stored, protected from light, in a desiccator.

9.9.2 Storage of spores in water

Purified spores are stored 1–2 mg dry weight/ml in cold deionized water, protected from light. Periodically (approximately once a week) the spores are centrifuged (10 000g, 10 min) and resuspended in cold deionized water.

9.9.3 Potential problems

For long-term storage of large amounts of spores, lyophilization is the method of choice, as it prevents spore germination, as well as growth of contaminating microorganisms. However, it is possible that lyophilization can alter some spore properties. Storing spores in cold water does not present the radical change in the spore environment given by lyophilization. However, contamination by bacteria or photosynthetic organisms can become a problem, as spores leak minute amounts of nutrients upon

spore population, with some 'superdormant' spores taking an hour or more.

Dormant spores appear bright by phase-contrast microscopy. When germinants are added, the process of germination may be observed as a progressive change from phase bright through phase grey to phase dark, accompanied by swelling to approximately twice the size of the dormant spore as water is absorbed (Gould, 1969). Vary & Halvorson (1965) used the term 'microlag' to define the length of time from the addition of the germinant to the first sign of phase darkening, and 'microgermination' to define the time from the end of microlag to completion of phase darkening of an individual spore. During the microlag the spores become committed to germination (Scott & Ellar, 1978b), after which point germination will still continue if the germinant is removed.

Sections of germinating spores viewed by electron microscopy reveal that before germination, and for some minutes following addition of germinants, the spore cortex is unstained and occupies a relatively large area between the core and the coat. Following the onset of germination the cortex becomes stained and assumes a striated appearance before shrinkage of the cortex and swelling of the core become apparent. Visible degradation of the coat follows that of the cortex (Santo & Doi, 1974; Sammons *et al.*, 1987).

Mutations at 13 chromosomal locations (*gerA-M*) give rise to spore germination mutants. They may be classified according to the germinant(s) in which the germination response is deficient (L-alanine and/or AGFK) and the stage at which germination is blocked. For example, some *ger* mutants fail to lose heat resistance and to undergo any of the later stages of germination, e.g. *gerA*, *B*, *D*, *F* (Moir *et al.*, 1979), while others lose heat resistance but do not complete degradation of the cortex, e.g. *gerM* (Sammons *et al.*, 1987). These mutant spores lose only part of their initial optical density and remain phase grey for relatively long periods after addition of germinants, before progressing to the phase-dark stage. In the case of the *gerE36* mutant a proportion of the spores in the population never reaches the phase-dark stage (Moir, 1981).

9.10.1 Germination media

Media which can be used in the isolation and characterization of germination mutants are given in Sections 9.10.1.a-9.10.1.d.

9.10.1.a PGA medium (Dring & Gould, 1971)

potato extract	4.0 g
yeast extract	4.0 g

storage. Similarly, spores stored in cold water will exhibit higher and higher percentages of germinated spores with time, and compounds released by germinating spores can stimulate other spores to germinate. While these problems can be minimized by weekly centrifugation of the spores to remove released nutrients, they cannot be completely eliminated. Spores can also be stored frozen or at -70°C in 50% glycerol. However, these methods are not recommended, as spores often germinate upon rewarming.

9.10 ISOLATION AND CHARACTERIZATION OF GERMINATION MUTANTS (contributed by Rachel Sammons, University of Birmingham, Birmingham, UK)

Spores can be induced to germinate by a variety of chemical and physical stimuli (Gould, 1969). Many of the chemical germinants are potential nutrients and are strain and species specific. Spores of *B. subtilis* germinate in response to L-alanine and related amino acids alone or to a mixture of L-asparagine, glucose, fructose and KCl (AGFK). Glucose, fructose and KCl increase the rate of germination in L-alanine and reduce the concentration of L-alanine required to achieve half the maximum germination rate (Sammons *et al.*, 1981). Such potentiation of germination by adjuncts is common for spores of a variety of *Bacillus* species. For example, inosine lowers the effective concentration of L-alanine for germination of spores of *B. cereus* T (Gould, 1969), whereas fructose and ribose stimulate germination of *B. macerans* spores in 2-phenylacetamide but not in asparagine (Sacks & Thompson, 1971). Spores of *B. megaterium* germinate in a variety of germinants, depending on the strain. *B. megaterium* KM germinates in response only to L-alanine and related amino acids (Scott & Ellar, 1978b), whereas other strains may be divided into two groups, one of which germinates in response to L-alanine + inosine and the other in response to D-glucose + ions (Rode, 1968). *B. megaterium* QMB1551 spores will germinate in L-alanine, proline, various sugars and inorganic potassium salts (Foerster & Foster, 1966).

Germination results in loss of heat and chemical resistance due to the degradation of the spore cortex and disruption of the coat and associated rehydration of the core. Loss of heat resistance is one of the earliest measurable changes which are observed following addition of germinants. Later changes include loss of optical density and the release of hexosamine-containing material from the cortex and dipicolinic acid from the core. Germination of an average spore of *B. subtilis* takes approximately 20-30 min from the addition of germinants to the commencement of RNA synthesis (Donellan *et al.*, 1965) but there is considerable variation within a

D-glucose 2.5 g
dH₂O to 1 l

Adjust pH to 7.4 with NaOH; autoclave.

9.10.1.b STBB medium

tryptose 10.0 g
beef extract 3.0 g
NaCl 5.0 g
Difco agar 10.0 g
H₂O to 1 l

Adjust pH to 7.2 with NaOH; autoclave.

9.10.1.c FTA medium

D-alanine 0.1 g
D-cycloserine 0.5 g
Difco agar 30.0 g
H₂O to 1 l

D-alanine and D-cycloserine are added as a powder to the autoclaved medium after the agar has cooled to 50 °C.

9.10.1.d Germination agar

Solutions needed

(1) K₂HPO₄·3H₂O 14.0 g
KH₂PO₄ 6.0 g
L-asparagine 0.5 g
Difco agar 15.0 g
dH₂O to 960 ml

The pH of this solution should be about 7.4. Aliquot into 192-ml volumes and autoclave.

(2) D-glucose 100 mg/ml
(3) D-fructose 100 mg/ml
(4) L-malate 100 mg/ml
(5) 2,3,5-triphenyltetrazolium chloride (Tzm) (Sigma) 100 mg/ml (protect from light; Tzm will quickly, within days, go pink when exposed to light).

Filter-sterilize solutions 2, 3, 4 and 5.

Germination agar

Autoclave 192 ml of solution 1; let cool to 60 °C. Add 2 ml of solutions 2, 3, 4 and 5; pour plates and store them protected from light.

9.10.2 Isolation of germination mutants

9.10.2.a Enrichment procedure

Mutagenized spores of a wild-type strain are washed free of any germinants and are then placed in conditions in which wild-type spores would normally germinate. After allowing sufficient time for most of the spores to germinate, those that have done so are killed by heat or chemical treatment. To allow 'superdormant' spores to germinate and to be killed, the germination process and killing treatment are repeated several times. The remaining dormant spores are then recovered by placing the spores in permissive conditions for germination and then plating for single colonies on an appropriate medium. Method 9.22 gives a procedure for enriching for heat-sensitive germination mutants.

The procedure may be modified to enrich for germinant specific mutants by initiating germination in one germinant and then shifting the spores to the permissive germinant. Mutants whose spores do not appear to germinate in both L-alanine and AGFK may be isolated by recovering ungerminated spores on rich medium such as Schaeffer's (Section 9.2.1.a), 2× SG (Section 9.2.1.b) or PGA (Section 9.10.1.a) on which they will eventually germinate. Any minimal agar can be used, such as TSSA, LGMA or GGMA (Table 2.3).

9.10.2.b Identification of germination mutants

These tests are based on the ability of germinating spores to reduce a colorless tetrazolium dye (2,3,5-triphenyltetrazolium chloride (Tzm)) to a red formazan derivative. The reduction process is presumably linked to reactivation of dehydrogenases which occurs at a relatively late stage in germination. Mutant colonies, whose spores do not reach this stage, do not reduce the dye and stay white (TzmW), while wild-type colonies become red (TzmR) as the spores germinate. The color of the colony may be diagnostic of the type of mutant; those which are blocked early in germination are TzmW, whereas those which lose heat resistance but fail to complete germination are Tzm pink. Two methods are used to identify uncharacterized mutants, the overlay test (Method 9.23) (Trowsdale & Smith, 1975), and the colony transfer test (Method 9.24) (Irie *et al.*, 1982). The colony transfer test distinguishes mutants which are able to germinate in L-alanine but not AGFK (e.g. *gerB* and *gerK*) from the wild type. These

mutants give a red color reaction in the overlay test identical to that of the wild type, presumably because the plates contain sufficient L-alanine to permit germination. With mutants which fail to germinate in both L-alanine and AGFK the colonies are white in both methods but the overlay test usually gives a clearer distinction from the wild-type. Note that sporulation mutants will also give TzmW colonies with both tests because the vegetative cells will be killed by the heat treatments. It is therefore essential to check that phase-bright spores are present by microscopy before assuming that the colony is that of a *ger* mutant. Because Cys⁻ mutants sporulate poorly, even on rich medium, it is advisable to add cysteine to the sporulation agar when testing the Tzm reaction of these mutants.

The procedure described in Method 9.24 is a quick and simple method for determining the germination phenotype of strains. Heating the transferred colonies on FTA agar will kill all vegetative cells and also heat-activate spores, while D-alanine will inhibit premature spore germination. D-cycloserine is included in FTA agar to inhibit alanine racemase. The developed filters can be quickly baked in an oven at 90 °C to stop the reaction and serve as a permanent record. It is advisable to include known Ger⁺ and Ger⁻ control colonies on each plate.

9.10.3 Preparation of spores for germination studies

Spores may be prepared by any of the methods previously described in this chapter (Section 9.8), but it must be noted that the method used may significantly affect the germination properties of the spores. It is essential that the properties of mutant spores be compared with those of the wild-type and/or parental strains prepared at the same time and under identical conditions, since spores may also vary from batch to batch and their germination response may alter with storage. It is necessary during the washing procedure to keep spore suspensions, centrifuge rotors, pots and water ice-cold at all times to avoid spore germination. For germination studies a spore suspension which is free of vegetative cells and has at least 95% phase-bright spores is required. Ten washes with water done over 1–2 days are usually sufficient to achieve this. Lysozyme may be added (100 µg/ml in 0.89% saline) after the fourth wash followed by 30 min of incubation with shaking at 37 °C and a further 10 washes to remove vegetative cells. However, the use of lysozyme should be avoided with uncharacterized mutants in case they are lysozyme sensitive. Spores are resuspended in distilled water and stored frozen at –20 °C in aliquots with an OD₅₈₀ of approximately 3.0.

9.10.4 Activation of spores for germination

Before measuring germination, spores are usually activated by heating in suspension in distilled water as above, for 30 min at 70 °C in aliquots as required. This reversibly increases the synchrony of germination of spores in the population and reduces the average microlag (Sammons *et al.*, 1981). Heat activation is not obligatory for germination of spores of most strains of *B. subtilis* but may be a requirement for spores of other *Bacillus* species. Spores of some thermophilic *Bacillus* species are activated by exposure to temperatures below their optimum growth temperature (Foerster, 1983).

9.10.5 Characterization of germination mutants

Germination mutants can be characterized according to their ability to germinate on one or a mixture of germinants. Germination features which can be followed include: loss in optical density (Method 9.25), loss in heat resistance (Method 9.26), excretion of dipicolinic acid (Scott & Ellar, 1978a) (Method 9.27), and release of hexosamine-containing material (Rondle & Morgan, 1955) (Method 9.28). These are usually measured during germination at 37 °C, but further information may be gained by employing lower or higher temperatures (e.g. 25 °C or 42 °C) and varying the germinant or adjunct concentrations. The concentration of spores is important since it may affect the germination response. In L-alanine more concentrated spore suspensions tend to germinate more slowly probably due to the presence of alanine racemase on the spore surface which converts L-alanine to D-alanine, a competitive inhibitor of germination in L-alanine. If D-alanine is used in inhibitor studies an inhibitor of the racemase, such as *o*-carbamyl-D-serine (OCDS; from International Minerals and Chemical Corporation, Terre Haute, Ind.) or D-cycloserines should also be present at the same concentration as the D-alanine.

Electron microscopy of germinating spores can also be a useful method for analyzing features of germination mutants (Sammons *et al.*, 1987; Sousa *et al.*, 1974; Glauert, 1974; Hayat, 1981). This analysis is described briefly in Method 9.29. However, full practical details are not given in this method, since anyone wishing to carry out electron microscopy is advised to seek expert advice on this technique.

9.10.6 Detection of germination at low germinant concentrations

While most procedures for studying spore germination use germinants at high concentration, it may be advantageous in some cases to monitor spore germination at very low germinant concentrations in order to detect germination in very small fractions of a spore population such as occurs at

low alanine concentrations ($< 6 \mu\text{M}$). This analysis exploits *B. subtilis* 168 strain UVSSP42-1 (Munakata & Ikeda, 1968) (BGSC 1A489), which produces spores which are extremely UV sensitive, but which acquire normal UV resistance upon germination (Munakata, 1974; Wang & Rupert, 1977; Irie *et al.*, 1984). Germination is estimated from survival after UV irradiation that kills almost all of the non-germinated spores and allows a majority of the germinated spores to survive. It has been suggested that this procedure may be potentially useful for enrichment for germination mutants or revertants which germinate under specified conditions as opposed to those which fail to germinate (Section 9.10.2). Spores which failed to germinate would be killed by UV treatment, whereas the mutants which did germinate would survive. The *ger* mutation could then be crossed out of the UV-sensitive mutant strain for further study.

9.11 ISOLATION OF SPORE OUTGROWTH MUTANTS

(contributed by Alessandro Galizzi, University of Pavia, Pavia, Italy)

The process of spore germination has traditionally been subdivided into three stages: activation, germination, and outgrowth. Activation (Section 9.10.4) is usually achieved by a heat shock, is reversible, and ensures that all spores proceed synchronously to the germination stage. Germination (Section 9.10) is characterized by the degradation of the spore cortex and other spore constituents, the release into the medium of dipicolinic acid, calcium and other ions, and the uptake of water. During outgrowth the degradative process is completed and the anabolic processes overtake the catabolic ones with the onset of RNA and protein synthesis being followed, much later, by DNA synthesis.

The distinction between germination and outgrowth is not absolute, and for obvious reasons all outgrowth mutants isolated have been conditional (temperature-sensitive) mutants. Outgrowth mutants have been operationally defined as mutants whose spores germinate but are unable to reach the first cell division in rich medium (nutrient broth supplemented with 0.5% glucose; Appendix 1) at the nonpermissive temperature, although they exhibit normal vegetative growth and sporulation at both the permissive and nonpermissive temperatures. The phenotypic block in all the mutants studied has agreed with the known temporal sequence of RNA, protein, and DNA synthesis during spore outgrowth. However, no correlation has been found between the genetic map position of outgrowth loci and the time of expression of their phenotypes. All temperature-sensitive outgrowth mutants studied recovered completely from their developmental block when the culture was shifted from the nonpermissive to the permissive temperature. None of the mutants could be cross-fed by

medium in which parental spores had been germinated. The phenotypes of several outgrowth mutants are repaired in the presence of 2% NaCl, 20% sucrose, or sublethal concentrations of distamycin A (Albertini *et al.*, 1979; Galizzi *et al.*, 1973; Tarchi-Fabbri *et al.*, 1981).

9.11.1 Mutagenesis and sporulation

Cells grown in nutrient broth are mutagenized by treatment with $100 \mu\text{g/ml}$ *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Adelberg *et al.*, 1965), washed, and resuspended at about 5×10^6 cells/ml in $2 \times$ SG medium (Section 9.2.1.b). The culture is incubated at 30°C until sporulation is complete; the spores are freed of growing cells as described in Section 9.8, and stored in cold distilled water. For other mutagenesis procedures, see Section 2.2.

9.11.2 Penicillin enrichment of outgrowth mutants

Mutagenized spores are suspended at 10^7 ml in nutrient broth and incubated at 47°C for 2.5 h. Penicillin G (Squibb) is then added to a final concentration of 500 units/ml and incubation continued for 4 h to kill any growing cells. The culture is then made to 500 units/ml in penicillinase (Sigma, Type I), incubated for 15 min at 47°C , and appropriate dilutions are spread on $2 \times$ SG plates (Section 9.2.1.b) to give 30–50 colonies per plate. Plates are incubated at 30°C until sporulation is complete (24–48 h), nonsporulated cells are killed by exposure of the plates to chloroform vapor for 10 min, plates are replica-plated on a rich medium plate (e.g. LB or nutrient agar; Appendix 1) and replicas are incubated for 12 h at 47°C . Spores from colonies not growing on replicas at 47°C are isolated and further characterized. Strains on which vegetative cells can grow and sporulate at 30°C and 47°C , and whose spores can germinate and multiply at 30°C but not at 47°C , are putative outgrowth mutants.

To classify them further, cleaned spores from individual strains are suspended in water at an OD_{600} of approximately 10, heat activated (70°C , 30 min) and then cooled. Spores are diluted 1/10 in each of two flasks containing 4.5 ml of a nutrient broth with 5 mM L-alanine, one incubated at 30°C and one at 47°C . The OD_{600} of both cultures is followed over the next hour. Strains whose spores lose $> 50\%$ of their initial optical density at 30°C but $< 10\%$ at 47°C are considered to be germination mutants. Strains whose spores lose OD_{600} at both 30°C and 47°C are outgrowth mutants.

9.11.3 Density gradient enrichment of outgrowth mutants

Cleaned mutagenized spores prepared as described in Section 9.11.1 are suspended at 10^9 /ml in 16.5% Urografin (see Section 9.8.4) and 3 ml of this

suspension are layered on a 20-ml linear gradient of from 33% to 75% Urografin. After centrifugation at 4°C for 30 min at 15 000 rev/min, all spores with a density greater than 1.25 g/ml are pooled, diluted four-fold with water, collected by centrifugation (10 min, 10 000g) and suspended in cold water. These spores are further screened as described in Section 9.11.2.

9.12 ISOLATION OF SPORULATION MUTANTS

Sporulation (Spo^-) mutants can be identified easily because of their colony phenotype. As mentioned earlier, sporulating (Spo^+) colonies will form brown pigmented colonies when grown on a sporulation agar; this can be Schaeffer's (Section 9.2.1.a), 2× SG (Section 9.2.1.b) or a minimal medium, e.g. LGMA, (Section 2.6.1.b; Table 2.3). Procedures for mutagenesis are given in detail in Chapter 2, and typical isolation procedures following mutagenesis involve diluting mutagenized cells to give isolated colonies on an agar plate. Colonies are allowed to incubate until the colony pigmentation has developed. Spo^- colonies will over time become easily visible against a background of pigmented Spo^+ colonies. These mutants can then be purified and characterized as described in this chapter.

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Characterization of Spores of *Bacillus subtilis* Which Lack Dipicolinic Acid

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Spores of *Bacillus subtilis* with a mutation in *spoVF* cannot synthesize dipicolinic acid (DPA) and are too unstable to be purified and studied in detail. However, the spores of a strain lacking the three major germinant receptors (termed Δ ger3), as well as *spoVF*, can be isolated, although they spontaneously germinate much more readily than Δ ger3 spores. The Δ ger3 *spoVF* spores lack DPA and have higher levels of core water than Δ ger3 spores, although sporulation with DPA restores close to normal levels of DPA and core water to Δ ger3 *spoVF* spores. The DPA-less spores have normal cortical and coat layers, as observed with an electron microscope, but their core region appears to be more hydrated than that of spores with DPA. The Δ ger3 *spoVF* spores also contain minimal levels of the processed active form (termed P_{41}) of the germination protease, GPR, a finding consistent with the known requirement for DPA and dehydration for GPR autoprocessing. However, any P_{41} formed in Δ ger3 *spoVF* spores may be at least transiently active on one of this protease's small acid-soluble spore protein (SASP) substrates, SASP- γ . Analysis of the resistance of wild-type, Δ ger3, and Δ ger3 *spoVF* spores to various agents led to the following conclusions: (i) DPA and core water content play no role in spore resistance to dry heat, desiccation, or glutaraldehyde; (ii) an elevated core water content is associated with decreased spore resistance to wet heat, hydrogen peroxide, formaldehyde, and the iodine-based disinfectant Betadine; (iii) the absence of DPA increases spore resistance to UV radiation; and (iv) wild-type spores are more resistant than Δ ger3 spores to Betadine and glutaraldehyde. These results are discussed in view of current models of spore resistance and spore germination.

Spores of *Bacillus* and *Clostridium* species normally contain $\geq 10\%$ of their dry weight as pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (21, 22, 39). This compound is synthesized late in sporulation in the mother cell compartment of the sporulating cell but accumulates only in the developing forespore (6, 36). The great majority of the spore's DPA is in the spore core, where it is most likely chelated with divalent cations, predominantly Ca^{2+} , although there are also significant amounts of Mg^{2+} and Mn^{2+} , with smaller amounts of other divalent cations (21, 22, 37, 39). In the first minutes of spore germination the DPA is excreted, along with the associated divalent cations (36, 37).

Since DPA is found only in dormant spores of *Bacillus* and *Clostridium* species and since these spores differ in a number of properties from vegetative cells, in particular in their dormancy and heat resistance, it is not surprising that DPA and divalent cations have been suggested to be involved in some of the spore's unique properties. There is some evidence in support of this suggestion, since mutants whose spores do not accumulate DPA have been isolated in several *Bacillus* species, and often these DPA-less spores are heat sensitive (1, 4, 25, 42, 43). Unfortunately, for some of these latter mutants the specific genetic lesion(s) giving rise to the DPA-less spore phenotype is not known. DPA is synthesized from an intermediate in the lysine pathway, and the enzyme that catalyzes DPA synthesis is termed DPA synthetase (6). In *B. subtilis* this enzyme is encoded by the two cistrons of the *spoVF* operon, which is expressed only in the mother cell compartment of the sporulating cell, the site of DPA synthesis. Mutants of *B. subtilis* likely to be

in or known to be in *spoVF* result in lack of DPA synthesis during sporulation, and the spores produced never attain the wet heat resistance of wild-type spores (1, 4, 6, 25). Unfortunately, it has been impossible to isolate and purify free spores from these *spoVF* mutants of *B. subtilis*, since the spores are extremely unstable and germinate and lyse during purification (B. Setlow and P. Setlow, unpublished results). This observation suggests that, at least in *B. subtilis*, DPA is needed in some fashion to maintain spore dormancy (7, 15), although the specific mechanism whereby this is achieved is not clear.

In addition to its possible roles in spore dormancy and resistance, DPA complexed with a divalent cation, usually Ca^{2+} , is an effective germinant of spores of almost all *Bacillus* and *Clostridium* species (15). These and other data have led to the suggestion that DPA may activate, possibly allosterically, some enzyme involved in spore germination (15). To date, this spore enzyme involved in spore germination has not been identified. However, DPA does allosterically modulate the activity of the germination protease (GPR) that initiates the degradation of the spore's depot of small, acid-soluble spore proteins (SASPs) during spore germination (14, 32). GPR is synthesized as an inactive zymogen (termed P_{46}) during sporulation, and P_{46} autoprocesses to a smaller active form (termed P_{41}) approximately 2 h later in sporulation. This conversion of P_{46} to P_{41} is stimulated allosterically by DPA, and only the physiological DPA isomer is effective (14, 32). The activation of this zymogen is also stimulated by the acidification and dehydration of the spore core, and together these conditions ensure that P_{41} is generated only late in sporulation, when the conditions in the spore core preclude enzyme action (14, 32). As a result, GPR's SASP substrates, which are synthesized in parallel with P_{46} , are stable in the developing and dormant spore. This is important for spore survival, as some major SASP (the α/β -type) are essential for the protection of spore DNA from a variety of

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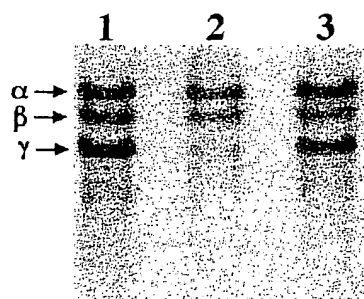


FIG. 3. Levels of SASP- α , - β , and - γ in spores. SASPs were extracted from purified spores of various strains, dialyzed, and lyophilized; aliquots were subjected to PAGE at low pH, and the gels were stained as described in Materials and Methods. The samples run on the various lanes (and the dry weight of the spores in the samples run) were as follows: lane 1, $\Delta ger3$ spores (1 mg); lane 2, $\Delta ger3 spoVF$ (0.6 mg); and lane 3, $\Delta ger3 spoVF$ spores prepared with DPA (1 mg).

prepared with DPA did contain a significant level of SASP- γ , although this level was a bit lower than in $\Delta ger3$ spores (Fig. 3, lanes 1 and 3).

Since the spores analyzed for SASP levels as described above remained for ~2 weeks at 12°C during preparation, it was of obvious interest to determine if developing spores of the $\Delta ger3 spoVF$ strain had never contained SASP- γ or had accumulated and then degraded this protein and, if the latter was the case, when the protein was degraded. Consequently, we analyzed SASP levels in sporulating cells during incubation at 37°C or subsequent incubation at 12°C (Fig. 4). Levels of SASP- α and - β were relatively constant once these proteins had accumulated in developing $\Delta ger3 spoVF$ spores, and SASP- γ was also present at high levels shortly after completion of SASP synthesis (Fig. 4, lane 1). However, levels of SASP- γ then began to decrease, and ~9 h later levels of this protein had fallen significantly and possibly fell even more after the culture had been harvested, washed with cold water, and incubated at 12°C. These data indicate that relatively normal levels of SASP- γ are accumulated by developing $\Delta ger3 spoVF$ spores but that the SASP- γ then disappears, presumably by degradation as sporulation and spore incubation proceeds.

Resistance of $\Delta ger3$ and $\Delta ger3 spoVF$ spores. The normal levels of SASP- α and - β in $\Delta ger3 spoVF$ spores suggested that the protection of spore DNA from damage by these proteins should be normal in $\Delta ger3 spoVF$ spores, and thus some aspects

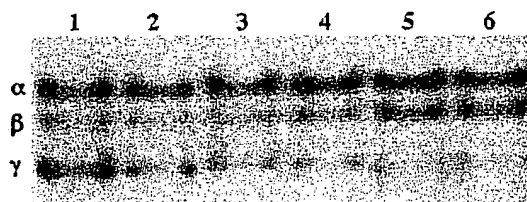


FIG. 4. Levels of SASP- α , - β , and - γ in sporulating cultures. Samples (10 ml) of strain FB108 ($\Delta ger3 spoVF$) sporulating in liquid 2 \times SG medium at 37°C were harvested, frozen, and lyophilized. After 36 h of growth, the remaining culture was harvested, washed several times with cold water, and resuspended in cold water. Again, aliquots equal to 10 ml of original culture were harvested, frozen, and lyophilized. Dry samples were disrupted; SASP was extracted; extracts were dialyzed, lyophilized, and redissolved; equal aliquots were subjected to PAGE at low pH, and the gel stained as described in Materials and Methods. The times (*t*) (in hours) in sporulation that the samples run in the various lanes were harvested were as follows: lane 1, *t*₄; lane 2, *t*₆; lane 3, *t*_{7.5}; lane 4, *t*_{13.5}; lane 5, *t*₂₄; and lane 6, *t*₁₆₀. Note that the last sample was incubated at 12°C for ~145 h. The migration positions of SASP- α , - β , and - γ are given on the left of the figure.

of spore resistance should be normal in these spores (39, 40). Indeed, previous work has shown that the spores formed by a *B. subtilis* strain with a mutation that is probably in *spoVF* are fully resistant to some chemical agents, including octanol and chloroform (1). However, these same spores were sensitive to a number of other chemicals and were also sensitive to wet heat (1). Given the known relationships between spore resistance and spore core hydration and mineral levels (11, 20, 40), it was of obvious interest to test the resistance of $\Delta ger3$ and $\Delta ger3 spoVF$ spores to a variety of agents. As seen previously and as expected based on the elevated level of core water in $\Delta ger3$ spores (1, 11, 28), the $\Delta ger3 spoVF$ spores were much less resistant to wet heat than were $\Delta ger3$ spores, while the latter spores had identical wet heat resistance to wild-type spores (Fig. 5A and data not shown). Spores of the $\Delta ger3 spoVF$ strain prepared with DPA exhibited an intermediate level of wet heat resistance (Fig. 5A). Although the $\Delta ger3 spoVF$ spores were significantly more sensitive to wet heat than were the wild-type spores, they were much more resistant than germinated spores or growing cells (<0.01% survival after 5 min at 70°C; data not shown). In contrast to the differences observed in the wet heat resistance of $\Delta ger3 spoVF$ and $\Delta ger3$ spores, both of these spores exhibited identical resistance to dry heat and were fully resistant to desiccation (Table 2 and data not shown). The resistance of these spores to dry heat and desiccation was identical to that of wild-type spores (data not shown) and much greater than that of growing cells (9, 35).

Analysis of resistance to hydrogen peroxide and formaldehyde gave results which were qualitatively similar to those with wet heat. The $\Delta ger3$ and wild-type spores exhibited identical resistance to formaldehyde and hydrogen peroxide (data not shown), while $\Delta ger3 spoVF$ spores were more sensitive and $\Delta ger3 spoVF$ spores prepared with DPA had intermediate levels of resistance (Fig. 5B and C). The UV resistance of $\Delta ger3$ spores was also identical to that of wild-type spores (data not shown), but $\Delta ger3 spoVF$ spores were more UV resistant than were $\Delta ger3$ spores, while $\Delta ger3 spoVF$ spores prepared with DPA had an intermediate level of resistance (Fig. 5D).

In contrast to wet heat, dry heat, UV, hydrogen peroxide, and formaldehyde, which had essentially identical efficiencies of killing of wild-type and $\Delta ger3$ spores, $\Delta ger3$ spores were significantly more sensitive to both the iodine-based disinfectant Betadine and to glutaraldehyde than were the wild-type spores (Fig. 6). The $\Delta ger3 spoVF$ spores exhibited decreased resistance to Betadine compared to that of $\Delta ger3$ spores, with $\Delta ger3 spoVF$ spores prepared with DPA exhibiting intermediate resistance (Fig. 6A). However, $\Delta ger3$ and $\Delta ger3 spoVF$ spores (with or without DPA) had identical resistance to glutaraldehyde (Fig. 6B).

DISCUSSION

Although DPA was discovered in spores of *Bacillus* species over 40 years ago, its specific function in spores has remained somewhat obscure. Correlations have been noted between spore wet heat resistance and DPA content (11, 22), but there are a number of observations indicating that DPA need not be essential for spore heat resistance. Thus, DPA plus associated divalent cations can be removed from the mature spores of several species by appropriate treatments, yielding spores with <1% of untreated spore DPA levels; these DPA-less spores retain a high level of wet heat resistance which is often similar to that of untreated spores (2, 11). Strikingly, these DPA-less spores of *Bacillus stearothermophilus* appeared to have more highly hydrated core regions than untreated spores yet still retained high wet heat resistance. The reasons for the wet heat

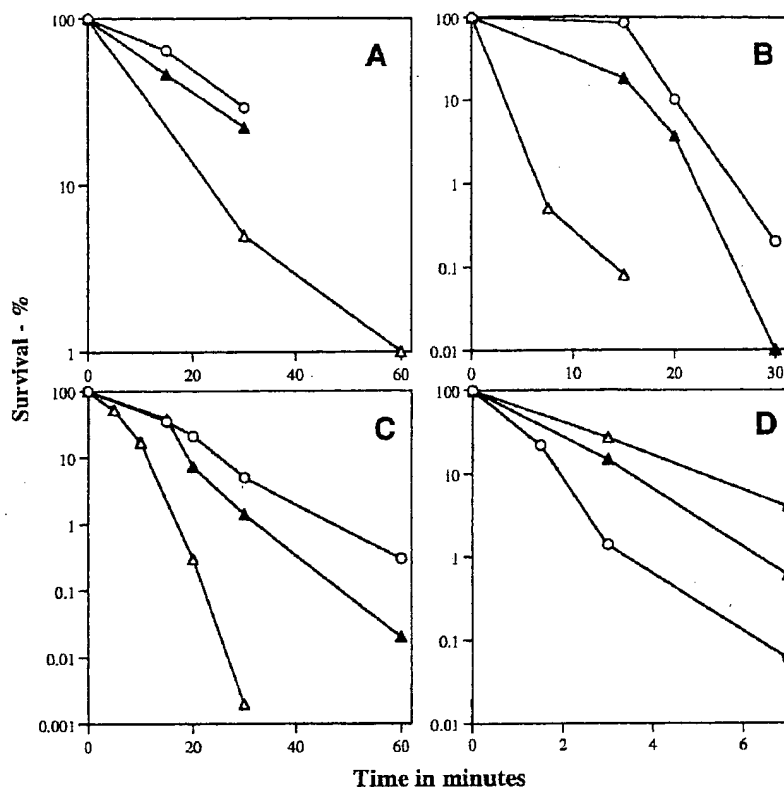


FIG. 5. Resistance of spores with or without DPA to heat (A), hydrogen peroxide (B), formaldehyde (C), or UV radiation (D). Spores were either heated at 85°C (○ and ▲) or 70°C (△) (A), incubated with 0.7 M hydrogen peroxide at room temperature (B), incubated with 0.3 M formaldehyde at room temperature (C), or UV irradiated at 150 J/m²·min (D), and the survival was measured as described in Materials and Methods. Symbols: ○, *Δger3* spores; △, *Δger3 spoVF* spores; ▲, *Δger3 spoVF* spores prepared with DPA. All experiments were repeated at least twice with essentially identical results.

resistance of these DPA-less and relatively demineralized spores are not clear, but these data indicate that DPA is not necessarily essential for spore wet heat resistance. However, it is possible that DPA accumulation during sporulation is required for the attainment of some state that is essential for full spore wet heat resistance. In support of this possibility, several studies, including the current one, have found that in *B. subtilis* the loss of the ability to synthesize DPA results in the production of wet heat-sensitive spores which exhibit increased core dehydration (1, 4, 6, 8). However, it is not clear if this effect is due only to a change in spore core hydration or also to the reduction in core mineralization which accompanies the loss of DPA from spores (12). Since spore core mineralization also plays a role in wet heat resistance (11, 20), it is certainly

possible that changes in both core hydration and mineral levels contribute to the loss of wet heat resistance of DPA-less spores.

Strains of *Bacillus cereus* and *Bacillus megaterium* with uncharacterized mutations that abolish DPA accumulation in spores also produce heat-sensitive spores, and in at least one case these spores appeared to have increased core hydration (42, 43). While the existence of these latter mutants would seem to support a role for DPA in spore heat resistance, there are several reports (11, 12) that the heat-sensitive DPA-less spores of *B. cereus* can be further mutated to give a strain that produces DPA-less but heat-resistant spores. Unfortunately, the genes responsible for these phenotypes are not known, and the heat-resistant phenotype of the DPA-less spores was extremely unstable (12). There is also an old report that heat-resistant DPA-less spores of *B. subtilis* had been isolated (42); unfortunately, there are almost no details available about this strain and the mutations which gave rise to this phenotype. Since addition of only very small amounts of DPA to *spoVF* cultures can result in production of at least some heat-resistant spores (1), possibly the mutants producing DPA-less, heat-resistant spores are actually oligosporogenous, and the heat-resistant spores arise from the acquisition of sufficient DPA by a fraction of spores, either through synthesis in the surrounding mother cell or from the culture medium (8). If only a fraction of the spore population in a culture acquired only a small amount of DPA, then analysis might well not detect significant DPA in the population as a whole.

TABLE 2. Spore resistance to dry heat and dessication^a

Treatment	% Survival		
	FB72 (<i>Δger3</i>)	FB108 (<i>Δger3 spoVF</i>) sporulated without DPA	FB108 (<i>Δger3 spoVF</i>) sporulated with DPA
Dessication ^b	96	92	98
Dry heat (120°C) for 30 min	26	23	24

^a Spores were produced and analyzed for dessication and dry-heat resistance as described in Materials and Methods.

^b Spores were freeze-dried once, and the viability was measured after rehydration.

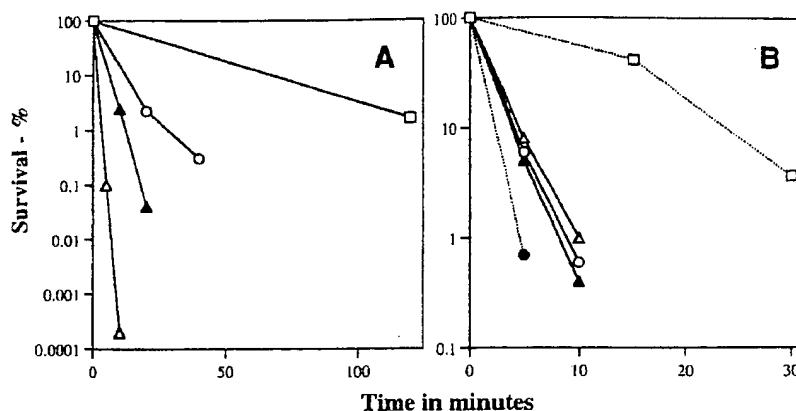


FIG. 6. Betadine and glutaraldehyde resistance of spores with or without DPA. Spores were incubated either with 85% Betadine at 37°C (A) or with 1.8% glutaraldehyde (□ and ●) or 0.5% glutaraldehyde (○, △, and ▲) at room temperature (B), and the survival was measured as described in Materials and Methods. Symbols: □, PS533 (wild-type) spores; ○ and ●, $\Delta ger3$ spores; △, $\Delta ger3 spoVF$ spores; ▲, $\Delta ger3 spoVF$ spores prepared with DPA. All experiments were repeated at least twice with essentially identical results.

The precise role of DPA in spores is still not clear; however, in *B. subtilis* specifically blocking DPA synthesis results in DPA-less spores with significantly less wet heat resistance than wild-type spores. The lack of DPA in these *B. subtilis* spores is accompanied by increased core hydration, and there are abundant data that this increase in core hydration should reduce spore wet heat resistance (11, 27) and, as shown here, it does. However, the DPA-less spores of *B. subtilis* are still significantly more wet heat resistant (and less hydrated) than are growing cells or germinated spores of this organism (26, 27, 40).

In addition to a role for DPA in spore wet heat resistance, two other roles have been proposed. One is to stabilize the dormant spore such that it does not germinate spontaneously (7, 16, 40). This appears to be the case for the *B. subtilis* spores studied in this work, since the $\Delta ger3 spoVF$ spores germinate spontaneously much more readily than the $\Delta ger3$ spores. Unfortunately, it is not clear at present either what is involved in "spontaneous" spore germination or how spore DPA could suppress this event. Surprisingly, it has also been reported that some DPA-less spores of *B. cereus*, *B. megaterium*, and *B. subtilis* germinate extremely poorly (12, 42). However, the mutation or mutations giving rise to the DPA-less spores of these strains are not known, and it is certainly possible that, in addition to a mutation blocking DPA synthesis or uptake, these strains have an additional mutation(s) suppressing spore germination, thus allowing the DPA-less spores of these strains to be isolated.

A second specific role for DPA is in allosterically stimulating the processing of GPR from P_{46} to P_{41} such that this processing only takes place very late in spore core maturation, when the core dehydrates; this dehydration also stimulates conversion of P_{46} to P_{41} (14, 32). The coupling of DPA accumulation and core dehydration with generation of active GPR ensures that minimal if any SASP degradation takes place in sporulation, maximizing the levels of these proteins, in particular the α/β -type SASPs which are essential for full spore DNA resistance and long-term spore survival (15, 40). This role of DPA and core dehydration in regulation of P_{46} processing is certainly consistent with the results presented here, since very little P_{46} is processed to P_{41} in spores of the $\Delta ger3 spoVF$ strain, and this processing is largely restored if these spores are prepared with DPA.

One result which seems to be at odds with the significantly reduced P_{41} generated in $\Delta ger3 spoVF$ spores is the degradation of the SASP- γ that is accumulated midway in sporulation. Previous work has shown that SASP are normally not degraded during sporulation (38), although this will take place, primarily with SASP- γ , if P_{41} is activated too early or in too high amounts or under conditions of too little core dehydration (12, 27, 32). Although very little P_{41} appears to be present in $\Delta ger3 spoVF$ spores, there could easily be ~5% of wild-type spore levels, and this would be more than enough to catalyze significant SASP- γ breakdown until sufficient core dehydration precludes further enzyme action. Alternatively, the degradation of SASP- γ in $\Delta ger3 spoVF$ spores might be catalyzed by proteases other than GPR, which slowly act on SASP- γ in the more hydrated core of these spores. One other possibility that deserves mention is that SASP- γ degradation may actually continue in the mature dormant spore. It is thought that enzyme action in the spore core is precluded by the low level of water in this region of the spore (39). However, the increased hydration of the $\Delta ger3 spoVF$ spore core may allow some low level of enzyme action. The fact that SASP- γ levels fall only somewhat slowly upon extended incubation of sporulating cells is suggestive of this possibility, but further detailed work on this and other enzyme-substrate pairs (39) in the core of DPA-less spores is needed.

As noted above, the increased hydration and the decreased mineralization of the core of $\Delta ger3 spoVF$ spores is consistent with their decreased resistance to wet heat (11). The decreased resistance of $\Delta ger3 spoVF$ spores to formaldehyde was also expected, since this agent kills spores by causing DNA damage in the spore core (17), and the rate of accumulation of this damage would be expected to be more rapid in a more hydrated spore core. Similarly, there are previous data indicating that within a species increasing core hydration is correlated with decreasing spore resistance to hydrogen peroxide (28), and this is consistent with the decreased hydrogen peroxide resistance of $\Delta ger3 spoVF$ spores. However, the precise target for hydrogen peroxide in spores is not known. It is also possible that the decreased mineralization of DPA-less spores plays some role in their decreased resistance to formaldehyde and hydrogen peroxide, but there are no data available on this point.

The normal resistance of $\Delta ger3 spoVF$ spores to dry heat and

dessication was also not unexpected, since these resistance properties are independent of core water content in *B. subtilis* spores and depend largely on the presence of α/β -type SASP (9, 35), and levels of these DNA protective proteins are normal in $\Delta ger3 spoVF$ spores. The presence of normal levels of α/β -type SASP in $\Delta ger3 spoVF$ spores also explains the UV resistance of these spores, since α/β -type SASPs are the major determinant of spore UV resistance (38, 40). The specific level of core dehydration plays very little if any role in spore resistance to UV radiation at 254 nm as shown previously (28), while DPA actually decreases spore UV resistance by acting as a photosensitizer (33); this latter point explains the increased UV resistance of $\Delta ger3 spoVF$ spores compared to that of $\Delta ger3$ spores.

All of the agents discussed above had identical efficiencies in killing wild-type and $\Delta ger3$ spores. In contrast, glutaraldehyde and the iodine-based disinfectant, Betadine, were much more effective in killing $\Delta ger3$ spores than wild-type spores. Both of these agents have been shown to kill spores in part by damaging the spore germination apparatus (3, 30, 41). The increased sensitivity of the $\Delta ger3$ spores to these agents may thus be due to the fact that CaDPA-triggered spore germination requires at least one protein which is in the spore's exterior layers (24) and thus is extremely sensitive to exogenous chemical agents (3, 24, 41). In contrast, wild-type spores appear to have at least one other pathway for triggering spore germination that does not require this sensitive protein (24). In support of this reasoning, the presence of DPA and various levels of core dehydration had no effect on spore resistance to glutaraldehyde, which is thought to block a very early step in spore germination. However, spore Betadine resistance was increased by DPA and increased core dehydration, suggesting that Betadine may also kill spores by inactivating some more interior protein(s).

While the analysis of the properties of the $\Delta ger3 spoVF$ spores has given us some insight into the role of DPA and core hydration in various aspects of spore resistance and biochemistry, the isolation of moderately stable spores of the $\Delta ger3 spoVF$ strain of *B. subtilis* also may prove useful in opening up other avenues of research. For example, DPA-less heat-sensitive spores of *B. cereus* have been used as a parent to isolate DPA-less heat-resistant spores (12). However, because of the relative paucity of genetics and techniques for genetic manipulation in *B. cereus*, the nature of the second mutation or mutations restoring heat resistance to these spores is not known. However, given the ease of genetic manipulation with *B. subtilis*, if the $\Delta ger3 spoVF$ strain can generate DPA-less but now heat-resistant spores, the analysis of the mutation giving this new phenotype should be straightforward and may give us much new insight into the mechanism of spore resistance to wet heat. This work is currently in progress.

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Chemical Germination of Native and Cation-Exchanged Bacterial Spores with Trifluoperazine

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The calmodulin antagonist trifluoperazine and its analog chlorpromazine, both amphipaths, induced chemical germination of spores of various species, as do many surfactants. Cation load can greatly influence this response. Calmodulin antagonism does not seem to be involved. A new fluorometric assay for dipicolinic acid based on the fluorescence of the dipicolinic acid chelate of Tb^{3+} was found to be simple and sensitive.

A possible role for a Ca-binding protein in the germination of bacterial spores has been suggested by a variety of studies (6, 7, 16, 17, 23, 26). Recent discoveries of calmodulinlike proteins in sporulating *Bacillus subtilis* (9) and in spores of *Bacillus cereus* (33) have stimulated interest in this concept and have led to investigations on the effects of calmodulin antagonists such as trifluoperazine (TFP; Y. T. Shyu and P. M. Foegeding, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, 1-8, p. 218) that show inhibitory effects of TFP on germination (11, 20). This report shows that TFP can induce chemical germination (21, 22) (a term suggested to describe germination events induced by many surfactants) because of its amphipathic (detergentlike) properties (2, 32) rather than because of possible effects on a calmodulinlike protein and that the exchangeable cation load of the spores can greatly influence such germination.

Spores of *B. megaterium* ATCC 10778 were found to be quite susceptible to TFP-induced germination (in the absence of any physiological germinants), and the large spores facilitated quantitative microscopy (Fig. 1). The chemical germination induced by TFP is distinguishable (12, 21) from physiological germination in that (i) it is lethal (viability drops in parallel with other germination events), (ii) darkening of spores usually proceeds only to the phase grey (5, 24, 30) stage (note the relatively small shift in A_{600}), and (iii) it can occur at 70°C (data not shown). Similar results were obtained with spores of *B. subtilis* 168 (19), *B. coagulans* 1491 (19), and *Clostridium cylindrosporium* HC-1 (29). Chlorpromazine, an analog of TFP, showed similar effects with spores of *B. megaterium* 10778. Chlorpromazine is also known for potent amphipathic properties (10, 14, 18, 32) and has been reported to inhibit physiological germination and prevent complete darkening (11). A number of Ca channel blockers were tested and found inactive; W-7, capable of reacting with Ca-binding proteins, showed only marginal activity. Presumably the changes seen in Fig. 1 result from the amphipathic nature of the TFP, not from its ability to react with Ca-binding proteins. Since germination appears to be checked at a late stage, as evidenced by the incomplete refractility changes and loss of viability, there does not appear to be a conflict with prior results (11; Shyu and Foegeding, Abstr. Annu. Meet. Am. Soc. Microbiol., 1989) demonstrating the inhibitory effects of TFP and chlorpromazine on normal germination in the presence of germinants. When spores of *B. megaterium* were titrated at pH 4 with HCl (26) to remove exchangeable cations and "reloaded" with various selected cations (1, 23, 26), Ca-loaded spores

(Ca-spores) were found to be quite resistant to TFP-induced germination but NH_4 -spores were more susceptible than the native spores or Na-spores (Fig. 2). Dodecylamine, unique among surfactants (12, 13, 22) in its high activity and its ability to mimic true germination, was tested with Ca- and NH_4 -spores of *B. megaterium*, and the Ca-spores were markedly less susceptible to germination. Native spores of *Bacillus macerans* B-171 (25, 28) were quite refractory to TFP and dodecylamine, but NH_4 -spores germinated to a considerable extent. However, it was necessary to titrate these spores at pH 3.2 to achieve adequate cation exchange, and reloading conditions were also altered (1 M, 18 h, 37°C) for maximum effectiveness when reloading with NH_4^+ (4). The only previous report suggesting an influence of cation load on surfactant-induced germination showed that endotrophically formed Sr- and Ba-spores were less susceptible to dodecylamine-induced germination than Ca-spores (8), but since the results paralleled the physiological germination of these spores, little note was taken of them. The work reported here suggests that exchangeable cation load may greatly influence susceptibility to chemical germination. Germination induced by dodecylamine appeared to be qualitatively similar to germination induced by TFP or chlorpromazine. In all the experiments reported above, dipicolinic

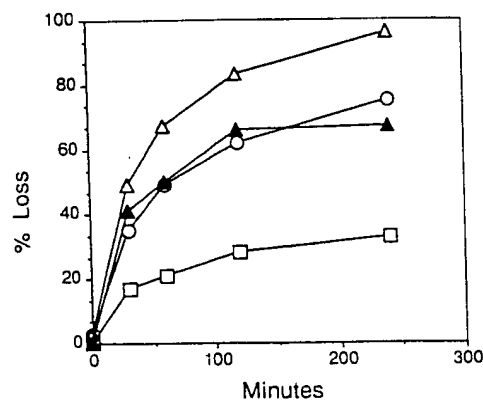


FIG. 1. Germination of spores of *B. megaterium* ATCC 10778 (prepared as explained in references 26 and 27) by TFP (100 μ M) in 50 mM KMOPS, pH 7.5. Symbols: \blacktriangle , spore refractility, i.e., percent nonrefractile spores (phase grey [$>90\%$] or dark spores) by phase microscopy; \triangle , percent DPA released (see text); \circ , viability of unheated spores as CFU on plate count agar (Difco); \square , A_{600} . The experiment was performed at 37°C.

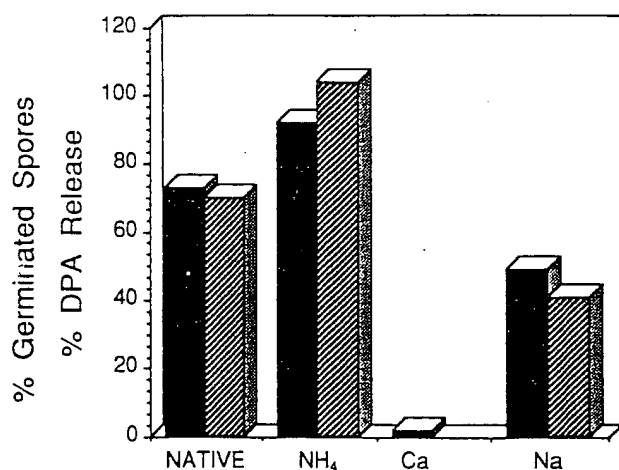


FIG. 2. TFP-induced germination (after 140 min at 37°C) of spores of *B. megaterium* ATCC 10778 reloaded with various cations. Solid bars, percent phase grey spores plus dark spores; hatched bars, percent total DPA released (methods were as explained in the legend to Fig. 1).

acid (DPA) was determined by a new test based on the formation of a highly fluorescent chelate of Tb^{3+} with DPA, with characteristic excitation and emission peaks (3). The test is performed by adding up to 2.0 ml of the sample to 1 ml of sodium citrate (1.0 M, pH 5.5) and 1 ml of $TbCl_3$ (1 mM), and emission is measured at 545 nm after excitation at 280 nm; a Turner model 430 spectrofluorometer was employed throughout. Standard curves were linear from 0 to 10 $\mu g/ml$ in the presence of 50 mM KMOPS (potassium-morpholine-propanesulfonic acid), the buffer used for these tests, and amounts of $<1 \mu g/ml$ were readily detected (31); the addition of TFP resulted in a slight change of slope and intercept, but the curve remained linear, so it was readily interpreted. This test is an inverted version of the assay used to determine Tb^{3+} by chelation with DPA (3); citrate was substituted for the acetate buffer to minimize interference by certain cations (3). Citrate invariably resulted in solutions with greater fluorescence (34). Inosine interferes, possibly because of strong absorbance at the excitation wavelength. This test is simple to perform and is considerably more sensitive than the commonly used Fe^{2+} colorimetric test (15), readily detecting DPA released in germination experiments designed for absorbance monitoring.

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Proteolytic Processing of the Protease Which Initiates Degradation of Small, Acid-Soluble Proteins during Germination of *Bacillus subtilis* Spores

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Degradation of small, acid-soluble spore proteins during germination of *Bacillus subtilis* spores is initiated by a sequence-specific protease called GPR. Western blot (immunoblot) analysis of either *Bacillus megaterium* or *B. subtilis* GPR expressed in *B. subtilis* showed that GPR is synthesized at about the third hour of sporulation in a precursor form and is processed to an approximately 2- to 5-kDa-smaller species 2 to 3 h later, at or slightly before the time of accumulation of dipicolinic acid by the forespore. This was found with both normal levels of expression of *B. subtilis* and *B. megaterium* GPR in *B. subtilis*, as well as when either protein was overexpressed up to 100-fold. The sporulation-specific processing of GPR was blocked in all *spoIII*, *-IV*, and *-V* mutants tested (none of which accumulated dipicolinic acid), but not in a *spoVI* mutant which accumulated dipicolinic acid. The amino-terminal sequences of the *B. megaterium* and *B. subtilis* GPR initially synthesized in sporulation were identical to those predicted from the coding genes' sequences. However, the processed form generated in sporulation lacked 15 (*B. megaterium*) or 16 (*B. subtilis*) amino-terminal residues. The amino acid sequence surrounding this proteolytic cleavage site was very homologous to the consensus sequence recognized and cleaved by GPR in its small, acid-soluble spore protein substrates. This observation, plus the efficient processing of overproduced GPR during sporulation, suggests that the GPR precursor may autoproteolyze itself during sporulation. During spore germination, the GPR from either species expressed in *B. subtilis* was further processed by removal of one additional amino-terminal amino acid (leucine), generating the mature protease which acts during spore germination.

Approximately 10 to 20% of the protein of spores of *Bacillus* species is degraded to amino acids in the first minutes of spore germination (21). The proteins degraded in this process are a group of small, acid-soluble spore proteins (SASP), and SASP degradation is initiated by a sequence-specific protease termed GPR (10, 18, 21); the gene coding for GPR (termed *gpr*) has been cloned and sequenced from both *Bacillus megaterium* and *Bacillus subtilis* (27). Expression of *gpr* begins only in the developing forespore in the third hour of sporulation at or slightly before the time of synthesis of its SASP substrates (11, 27). Studies of GPR from *B. megaterium* (11) have shown that it is a tetramer of identical subunits which are first synthesized as an apparent 46-kDa polypeptide (termed P_{46}), which is converted to an apparent 41-kDa species (termed P_{41}) ~2 h later in sporulation. During the first minutes of spore germination, P_{41} is converted to an apparent 40-kDa species (termed P_{40}); P_{40} is then slowly degraded to completion in an energy-requiring process as spore germination and outgrowth proceed. The processing of P_{46} to P_{41} appears to be an important step in the regulation of GPR activity, because the tetrameric enzyme composed of P_{46} is inactive both in vivo and in vitro (7). In contrast, the P_{41} form of the enzyme is active in vitro but not in vivo (7). The significance of the P_{41} to P_{40} conversion is not clear. At present, the exact processing steps involved in conversion of P_{46} to P_{40} are not known. However, purified P_{40} from *B. megaterium* lacks 16 amino-terminal residues predicted from the coding gene's sequence (27). This plus other data (11) strongly suggest that the

conversion of P_{46} to P_{40} requires at least one amino-terminal proteolytic cleavage.

The processing of P_{46} to P_{41} takes place at a time in sporulation when the developing spore is becoming dormant and heat resistant (11, 27). This finding, plus the suggestion that the regulation of the P_{46} to P_{41} conversion may be a mechanism for regulating GPR activity, makes understanding this conversion of interest, in particular with regard to the identification and understanding of the regulation of the processing enzyme(s). In addition, while we now have some knowledge of GPR processing during sporulation, this information is available only with *B. megaterium* (11); clearly comparable information in *B. subtilis* would be valuable. Consequently, in this report we present studies of GPR processing in *B. subtilis*, including the processing of both *B. megaterium* and *B. subtilis* GPR expressed at normal or high levels, as well as the *spo* gene dependence of this processing and the location of the processing sites in the GPR sequence.

MATERIALS AND METHODS

Bacteria and plasmids. The bacteria and plasmids used in this work are listed in Table 1. The *B. megaterium gpr* gene was excised from plasmid pPS740 by cleavage with *HincII* and *HindIII*, releasing a 1.6-kb fragment containing the coding sequence and promoter (27). The ends of this fragment were filled in with the large fragment of *Escherichia coli* DNA polymerase I and ligated with the 7.8-kb fragment from plasmid pPS1393 (a hybrid plasmid between plasmids pUC19 and pUBB which has a *B. subtilis* replication origin [29]) which had been cut with *HpaI* and from which the 0.13-kb fragment had been removed. The resulting ligation

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TABLE 1. Bacteria and plasmids used

Bacteria or plasmid	Genotype or phenotype	Source or reference
<i>E. coli</i> RR101		Lab stock
<i>B. subtilis</i> PS69	<i>spoVA89 trpC2</i>	BGSC ^a
PS356	Δ <i>sspA</i> Δ <i>sspB</i> <i>trpC2</i> Cm ^r	15
PS604	<i>spoIIIC94 trpC2</i>	BGSC
PS683 (MO428)	<i>spoIIIGΔ1 trpC2</i>	P. Stragier
PS832	<i>trp</i> ⁺ revertant of strain 168	Lab stock
PS1029	<i>gpr::pJF751</i> Cm ^r <i>gpr</i>	18
PS1208	<i>spoIIAC1 trpC2</i>	J. Errington
PS1586	<i>spoIVB105</i>	R. Losick
PS1675	<i>rpoB2 spoVD156 trpC2</i>	BGSC
PS1676	<i>spoVE85 trpC2</i>	BGSC
PS1677	<i>metC3 spoIVG25 tal-1</i>	BGSC
PS1732	<i>spoVF224 trpC2</i>	P. Piggot
PS1762	<i>spoVIA513</i>	BGSC
PS1763	<i>gerE36 leu-2</i>	BGSC
PS1850	<i>spoIVA::Tn917</i> H194 MLS ^r	R. Losick
PS1853	<i>spoIVF</i> Δ <i>AB::cat</i> Cm ^r	R. Losick
Plasmid		
pPS740	<i>B. megaterium gpr</i> in pUC12, Amp ^r	26, 27
pPS1542	<i>B. subtilis gpr</i> in pUC12, Amp ^r	26
pPS1393	Hybrid plasmid between pUBB and pUC19, Amp ^r Km ^r	29
pPS1669	pUBB with 0.13-kb <i>HpaI</i> fragment replaced with 1.6-kb fragment containing <i>B. megaterium gpr</i> , Km ^r	This work
pPS1671	pUBB with 0.13-kb <i>HpaI</i> fragment replaced with 1.4-kb fragment containing <i>B. subtilis gpr</i> , Km ^r	This work
pPS1673	ptrpBG1 with 0.9-kb <i>HindIII</i> fragment replaced with 1.6-kb fragment containing <i>B. megaterium gpr</i> , Amp ^r Cm ^r	This work
ptrpBG1	Amp ^r Cm ^r	22

^a BGSC, *Bacillus* Genetic Stock Center.

mix was cut with *Bam*HI to remove plasmid pUC19, religated, and used to transform *B. subtilis* PS832 to kanamycin resistance; one clone carrying a plasmid giving 2.2- and 4-kb fragments upon digestion with *Xba*I was identified. In this plasmid (pPS1669), the *B. megaterium gpr* gene retains its own promoter and is just downstream of the strong, sporulation-specific promoter of the *sspB* gene (14, 16, 29). The 1.6-kb fragment carrying the *gpr* gene described above, in which the *Hind*III end had been filled in, was ligated with the 6.3-kb fragment from plasmid ptrpBG1 (22) (which lacks a *B. subtilis* replication origin), which had also been digested with *Hind*III and the ends of which had been filled in with the large fragment of *E. coli* DNA polymerase I. The ligation mix was used to transform *E. coli* RR101 to ampicillin resistance, and one clone carrying a plasmid (pPS1673) which gave 4.6- and 7.6-kb fragments on digestion with *Pst*I and *Xba*I was identified. Plasmid pPS1673 contains the *B. megaterium gpr* gene with its own promoter, flanked by front and back sections of *amyE*. After linearization of this plasmid with *Pst*I, use of the DNA to transform *B. subtilis* to a chloramphenicol-resistant, amylase-negative phenotype resulted in integration of the *B. megaterium gpr* gene plus its promoter at the *amyE* locus (22). Southern blot analysis of representative transformants confirmed the expected integration (data not shown).

The *B. subtilis gpr* gene was excised from plasmid pPS1542 by cleavage with *Bam*HI. This digestion releases a 1.4-kb fragment containing the *gpr* coding sequence but lacking the gene's own promoter (26, 27). After the ends of the latter fragment were filled in with the large fragment of *E.*

coli DNA polymerase I, it was ligated with the 7.8-kb *Hpa*I fragment of plasmid pPS1393; the ligation mix was cut with *Bam*HI to remove plasmid pUC19, religated, and used to transform *B. subtilis* 168 to kanamycin resistance. One clone was found to contain a plasmid which released 0.8- and 5.5-kb fragments on digestion with *Sst*I. This plasmid was termed pPS1671 and carries the *B. subtilis gpr* gene under control of the strong, sporulation-specific promoter of the *sspB* gene.

Growth, sporulation, spore germination, and DNA isolation. *B. subtilis* strains were grown and sporulated at 37°C in 2× SG medium (6) with appropriate antibiotics (chloramphenicol, 3 µg/ml; kanamycin, 10 µg/ml). The approximate time of initiation of sporulation in these cultures was determined as described previously (17). Spores were purified as described previously (15), and all spores used in this work were free (>98%) of sporulating cells, germinated spores, and cell debris. Spore germination followed a heat shock (70°C; 30 min) of spores (~5 mg [dry weight]/ml) in water. After cooling in ice, spores were germinated at an optical density at 600 nm of 1.0 at 37°C in 2× YT medium plus 4 mM L-alanine (9, 18). Chromosomal and plasmid DNA was isolated from *B. subtilis* strains as described previously (15, 17). *B. subtilis* strains were made competent and transformed, and transformants were selected as described previously (2, 17).

Analytical procedures. For analysis of GPR during sporulation or germination, samples of culture (10 to 20 ml from sporulating cells; 25 to 100 ml from germinating spores) were chilled by addition of ice and harvested by centrifugation (10

min; 10,000 × g); the pellet was washed with 10 to 20 ml of 0.15 M NaCl, frozen, and lyophilized. The dry pellet was disrupted in a dental amalgamator (Wig-L-Bug) with glucose crystals as the abrasive (11); eight 1-min periods of shaking sufficed to break even dormant spores. The dry powder was extracted with 0.8 ml of cold 50 mM Tris-HCl (pH 7.4)–3 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride–20% glycerol for ~1 h and centrifuged in a Microfuge, and the supernatant fluid was stored frozen. Aliquots (2 to 30 µl) of the supernatant fluid were boiled with sodium dodecyl sulfate (SDS)–polyacrylamide gel sample buffer and run on SDS–10% polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose paper (11, 18). These papers were then treated as described previously, with rabbit anti-*B. megaterium* GPR as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G as the second antibody (7, 11). The preparation of the anti-*B. megaterium* P₄₀ serum was described previously (10); preparation of anti-P₄₆ serum is described below. The anti-P₄₆ serum had a 10- to 50-fold-higher titer against *B. subtilis* GPR on Western blots (immunoblots) than did the anti-P₄₀ serum. Consequently, the anti-P₄₆ serum was routinely used for analysis of *B. subtilis* GPR expressed at normal levels in *B. subtilis* and in some experiments monitoring normal levels of expression of *B. megaterium* GPR. The secondary antibody on the paper was detected either colorimetrically (18, 27) or via chemiluminescence with the substrate Lumi-Phos 530 (Boehringer Mannheim). The colorimetric method was used for detection of *B. megaterium* GPR; the chemiluminescence method was routinely used for detection of *B. subtilis* GPR, as we found this method more sensitive than the colorimetric method.

In some experiments, the proteins separated by SDS-polyacrylamide gel electrophoresis of extracts from 2 to 5 mg of spores overexpressing *B. megaterium* or *B. subtilis* GPR were transferred to polyvinylidenedifluoride paper (Immobilon; Millipore Corp.) and stained lightly with Coomassie blue, and GPR bands were cut out and sequenced in an automated gas phase sequencer as described previously (25).

Aliquots (1 to 2 ml) of sporulating cells were extracted and assayed for glucose dehydrogenase and dipicolinic acid (DPA) as described previously (14, 16). SASP were extracted from spores and quantitated as described previously (16).

Purification of *B. megaterium* P₄₆ and preparation of anti-P₄₆ antisera. *B. megaterium* P₄₆ was obtained from *E. coli* PS740 which carries the *gpr* gene on plasmid pUC12 under control of the plasmid's *lacZ* promoter (27); this strain makes 5 to 10% of its soluble protein as P₄₆ (26). One liter of this strain was grown overnight at 37°C in 2× YT medium (9) plus ampicillin (50 µg/ml) to an optical density at 600 nm of ~7.0. The cells were harvested by centrifugation (10 min; 10,000 × g), suspended in 300 ml of 25 mM Tris-HCl (pH 7.4)–5 mM CaCl₂ (buffer A), disrupted with lysozyme, and centrifuged for 45 min at 50,000 × g to remove unbroken cells, cell debris, and most chromosomal DNA. The supernatant fluid was treated with streptomycin sulfate to remove nucleic acids (10), and protein precipitating between 0 and 60% saturation of ammonium sulfate was isolated, dissolved in 20 ml of buffer A, and dialyzed against two 1-liter changes of buffer A containing 20% glycerol. After adjusting the solution to 0.1 M NaCl, the extract was run on a DEAE-Sephadex column as described previously for *B. megaterium* P₄₀ purification (10), and the P₄₆-containing fractions were detected by SDS-polyacrylamide gel electrophoresis. The

pooled material was further purified by chromatography on DEAE-cellulose as described previously (10). Combination of the peak fractions eluting from the latter column gave ~75 mg of P₄₆ that was >98% pure when run on SDS-polyacrylamide gel electrophoresis.

Purified P₄₆ was used to make antisera in rabbits, using a regimen similar to that described previously (10) but with three to five times as much protein per rabbit. The antiserum was prepared by East Acres Biologicals, Longmeadow, Mass.

RESULTS

GPR processing during sporulation. Previous work has indicated that GPR synthesis from the *gpr* gene's own promoter begins during sporulation slightly before synthesis of glucose dehydrogenase and well before synthesis of DPA (11, 27). Analysis of *B. megaterium* GPR synthesis driven by the *gpr* promoter from a single-copy gene in *B. subtilis* was consistent with these results, as GPR levels ≥50% of maximum were obtained when the level of glucose dehydrogenase was only 32% of maximum (Fig. 1A, lane 2). Similar results were obtained when expression of the chromosomal copy of *B. subtilis gpr* was measured (Fig. 1B, lane 1; and data not shown). Note that while the initially synthesized *B. megaterium* GPR (termed P₄₆) ran as an apparent 46-kDa species, the initially synthesized *B. subtilis* GPR (also termed P₄₆) migrated as an apparent 40-kDa species as noted previously (Fig. 1A,B) (18). Two to three hours after the initial appearance of GPR, the protein was converted to a smaller form (termed P₄₁), the apparent sizes being 41 kDa for *B. megaterium* GPR and 38 kDa for *B. subtilis* GPR (Fig. 1A and B) (18). For GPR of both species, the time of this initial processing step was well after accumulation of glucose dehydrogenase and at or slightly before the accumulation of DPA by the developing forespore (Fig. 1A and B). For both GPRs, a significant amount of the P₄₆ (20 to 40%) was not processed to P₄₁ (Fig. 1A and B, lanes 5); samples taken as long as 24 h after the start of sporulation or from cleaned dormant spores gave distributions of P₄₆ and P₄₁ similar to those found 12 h into sporulation (*t*₁₂) (data not shown). Previous work showed that significant amounts of P₄₆ (~25% of the amount synthesized) are also not processed to P₄₁ during sporulation of *B. megaterium* (11).

When GPR synthesis from a gene on a multicopy plasmid was driven by the strong *sspB* promoter, GPR synthesis appeared to be slightly later in sporulation (relative to glucose dehydrogenase synthesis) than when expression was driven by the *gpr* gene's own promoter (Fig. 2A, and B; cf. Fig. 1). This was not surprising, because the *sspB* gene is normally transcribed ~30 min after *gpr* (27). Despite synthesis of up to 100-fold more GPR from the *sspB* promoter than from the *gpr* promoter (see below), the initial processing to P₄₁ again took place at or slightly before the time of DPA accumulation by the forespore. As was found when cells expressing normal levels of GPR were analyzed, a significant amount (30 to 50%) of the overexpressed GPR was not processed to P₄₁ during sporulation (Fig. 2A and B; and see below). While forms of *B. megaterium* GPR other than P₄₁ and P₄₆ were seen during sporulation in this experiment (Fig. 2A), these did not appear in the mature spore. Possibly these other forms were generated in cells in which sporulation was aberrant and were detected because of the huge amount of GPR produced.

Analysis of spores overexpressing *B. megaterium* GPR indicated that P₄₆ and P₄₁ were the major protein bands

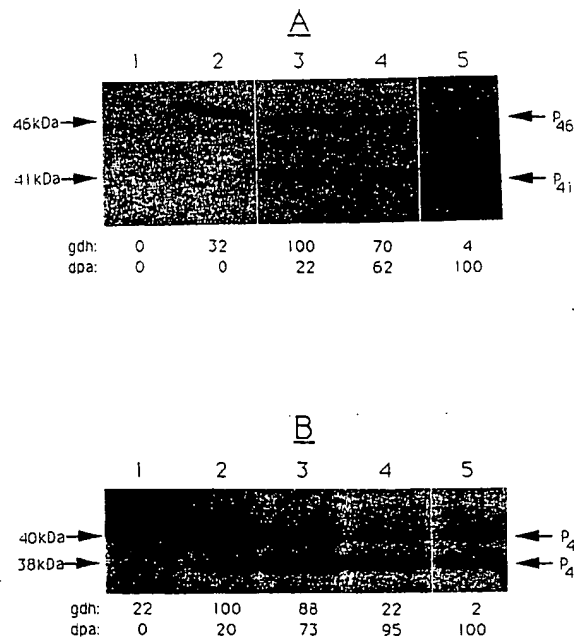


FIG. 1. Analysis of (A) *B. megaterium* and (B) *B. subtilis* GPR expressed from a single-copy gene. Strain PS832 (A) with plasmid pPS1673 (*B. megaterium gpr*) integrated at the *amyE* locus or (B) with no plasmid was grown and sporulated in 2× SG medium, and 20-ml samples were isolated, disrupted, and extracted as described in Materials and Methods. Aliquots (20 μ l) were run on SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose, and GPR was detected with anti-*B. megaterium* P₄₀, using either the colorimetric (A) or the chemiluminescence (B) detection method. The labeled arrows on the right give the migration position of the initially synthesized GPR (termed P₄₆) and the initial processed product (termed P₄₁). These designations were originally made on the basis of apparent molecular weights (arrows on the left) of *B. megaterium* GPR determined with reference to molecular weight standards. While we also use these designations for *B. subtilis* GPR, the initially synthesized GPR from this species actually has an apparent molecular weight of 40,000, and its processed form has a molecular weight of ~38,000 (arrows on the left) determined with reference to molecular weight standards. The identities of the bands noted as derived from *gpr* genes were confirmed by their absence in either strain PS832 (which lacks *B. megaterium* GPR) or PS1029 (in which *gpr* has been interrupted [18]). Aliquots of each culture were also analyzed for glucose dehydrogenase (gdh) and DPA (dpa). The results of these analyses are presented as the percentage of the maximum amount accumulated. The decrease in glucose dehydrogenase after the maximum is reached is due to the acquisition of lysozyme resistance by the developing spore. The samples run in the various lanes were harvested at various times after the initiation of sporulation. These times were as follows: (A) lane 1, 3 h; lane 2, 4 h; lane 3, 6 h; lane 4, 8 h; and lane 5, 12 h; (B) lane 1, 4 h; lane 2, 5 h; lane 3, 6 h; lane 4, 8 h; and lane 5, 12 h. For the culture analyzed in panel B, no detectable GPR was present in a 2-h sample (data not shown).

detected when the soluble fraction of a dormant spore extract was run on SDS-polyacrylamide gel electrophoresis and the proteins were transferred to Immobilon paper (Fig. 3, lane 1). Similar results were obtained when gels were stained directly (data not shown). Comparison of the GPR band intensities in this extract with those in extracts from spores of PS832 (Fig. 3, lane 2) (in which *B. subtilis* GPR makes up ~0.02% of total spore protein [11, 18]) indicated that the overexpressed *B. megaterium* GPR makes up 2 to 5% of total spore protein. (Note that spore coat, cortex,

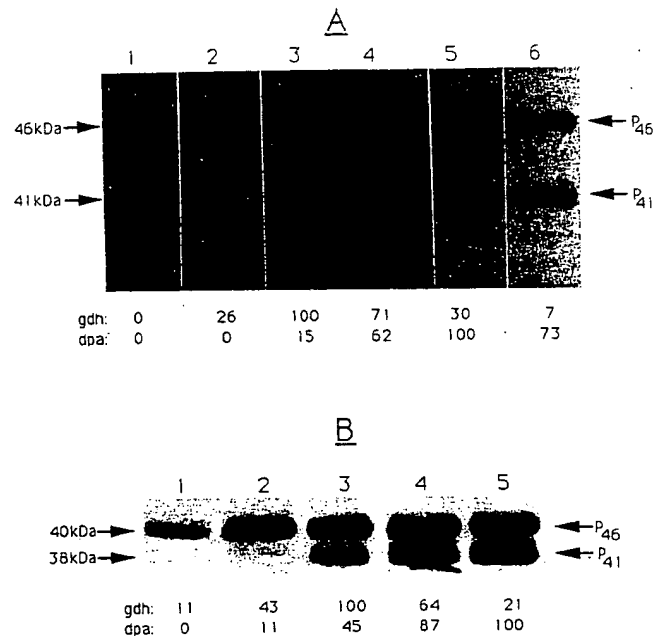


FIG. 2. Analysis of (A) *B. megaterium* and (B) *B. subtilis* GPR expressed from a multicopy plasmid. Strain PS832 carrying either (A) plasmid pPS1669 (*B. megaterium gpr*) or (B) plasmid pPS1671 (*B. subtilis gpr*) was grown and sporulated, and 10-ml samples were isolated, disrupted, and extracted as described in Materials and Methods. Aliquots (2 μ l in panel A, 10 μ l in panel B) were run on SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose, and GPR was detected with anti-*B. megaterium* P₄₀, using either the colorimetric (A) or the chemiluminescence (B) detection method. The arrows on the right denote the migration position of the originally synthesized GPR (P₄₆) and its processed form (P₄₁). The arrows on the left give the apparent molecular weights of these species. Values for the percentage of maximum glucose dehydrogenase (gdh) or DPA (dpa) accumulated at each time point are given below the lanes. The decrease in glucose dehydrogenase after the maximum is reached is due to acquisition of lysozyme resistance by the developing spore. The times after the initiation of sporulation at which samples run in various lanes were harvested are as follows: (A) lane 1, 2 h; lane 2, 4 h; lane 3, 6 h; lane 4, 8 h; lane 5, 12 h; and lane 6, 24 h; (B) lane 1, 4 h; lane 2, 5 h; lane 3, 6 h; lane 4, 8 h; and lane 5, 10 h. For the culture analyzed in panel B, no detectable GPR was present in a 2-h culture (data not shown).

ribosomal proteins, and SASP are not seen on this gel.) A similar analysis of the amount of *B. subtilis* GPR in spores of strain PS832 carrying plasmid pPS1671, and thus overexpressing *B. subtilis* GPR, gave a value of 0.2 to 0.5% (data not shown). The reason(s) for the difference in the maximum amount of *B. megaterium* and *B. subtilis* GPR accumulated in spores is not clear.

GPR synthesis and processing in *spo* mutants. Analysis of GPR levels in various *spo* mutants of *B. subtilis* showed that when the *B. megaterium gpr* gene was under the control of its own promoter, GPR was not produced by a *spoIIAC* mutant, but was produced in a *spoIIIG* mutant (Fig. 4A, lanes 1 and 3; Table 2). This is consistent with previous studies which indicate that most *gpr* transcription is initiated by RNA polymerase containing σ^F , the product of the *spoIIAC* gene, not by RNA polymerase containing σ^G , the product of the *spoIIIG* gene (27). Interestingly, the level of *B. megaterium* GPR produced in the two *spoIII* mutants tested was higher than that in a *spo*⁺ strain (Fig. 4A, lanes 2, 3, and 6). For the *spoIIIC* mutant comparable effects on



FIG. 3. Level of overexpressed *B. megaterium* GPR in spores. Cleaned spores (5 mg, dry weight) of *B. subtilis* PS832 with (lane 1) or without (lane 2) plasmid pPS1669 (high *B. megaterium* GPR expression) were disrupted and extracted as described in Materials and Methods. Aliquots (10 μ l) were run on an SDS-10% polyacrylamide gel, proteins were transferred to Immobilon paper, and the paper was stained lightly with Coomassie blue. The horizontal arrows denote the positions of *B. megaterium* P₄₆ and P₄₁.

other forespore-specific genes have been observed (14). In the case of the *spoIIIG* mutant, the overexpression of *B. megaterium* GPR could be due to the location of the *gpr* gene in the *amyE* locus; previous work has indicated that some

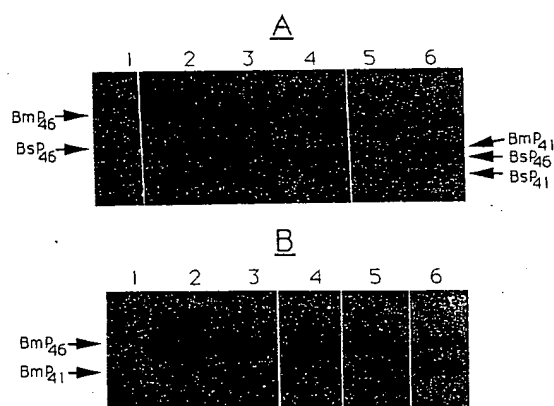


FIG. 4. Synthesis and processing of GPR in *spo* mutants. (A) *B. subtilis* strains with plasmid pPS1673 (normal level of *B. megaterium* GPR produced) were grown and sporulated, and 20-ml samples were harvested 12 h after the start of sporulation (t_{12}), disrupted, and extracted as described in Materials and Methods. Aliquots (20 μ l) were run on SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose, and GPR was detected with anti-*B. megaterium* P₄₆, using the colorimetric method. Horizontal arrows denote the positions of *B. megaterium* (Bm) or *B. subtilis* (Bs) P₄₆ and P₄₁. The identity of the *B. subtilis* GPR was established by its absence in strain PS1029 (*gpr*). The relevant genotypes for the strains from which samples were run in various lanes are as follows: 1, *spoIIAC*; 2, *spoIIIC*; 3, *spoIIIG*; 4, *spoVA*; 5, *spoVF*; 6, *spo*⁺. (B) *B. subtilis* strains with plasmid pPS1669 (overexpressed *B. megaterium* GPR) were analyzed as described above except that 10-ml samples were harvested, 2- μ l samples were run on the gel (except 10 μ l was run in lane 3), and anti-*B. megaterium* P₄₀ was used. The endogenous *B. subtilis* GPR is not seen on this gel. The genotypes of the strains from which extracts were run on the various lanes are the same as for panel A.

forespore-specific genes at the *amyE* locus exhibit anomalous expression in some *spo* mutants (24). The detection of GPR in this experiment (Fig. 4A) was with anti-*B. megaterium* P₄₆ as the primary antibody. Since this antiserum detected *B. subtilis* P₄₆ well, the P₄₆ from both *B. megaterium* and *B. subtilis* was detected on the same gel (Fig. 4A). Synthesis of *B. subtilis* P₄₆ was also abolished by a *spoIIAC* mutation, but levels of *B. subtilis* P₄₆ accumulated in a *spoIIIG* mutant were comparable to those in a *spo*⁺ strain (Fig. 4A, lanes 1, 3, and 6; Table 2). In contrast, when a promoterless *B. subtilis* *gpr* gene was overexpressed under control of the *sspB* promoter, which is recognized by RNA polymerase containing σ^G , the elevated level of GPR was abolished by a *spoIIIG* mutation (Table 2). With *B. megaterium* GPR overexpressed using the *sspB* promoter, GPR synthesis was abolished by a *spoIIAC* mutation and reduced >10-fold by a *spoIIIG* mutation (Fig. 4B, lanes 3 and 6; note large amount of *spoIIIG* mutant extract analyzed) (Table 2). Presumably the residual GPR synthesis in the latter strain is driven by the *B. megaterium* *gpr* gene's own promoter. Except as noted above, the synthesis of GPR from either the *gpr* or the *sspB* promoter was relatively unaffected by *spoIV*, -V, or -VI mutations (Fig. 4A and B; Table 2). However, the processing of GPR during sporulation was blocked by all *spoIII*, -IV, and -V mutants tested, but not by a *spoVI* or a *gerE* mutation or by deletion of genes coding for major SASP (Fig. 4A and B; Table 2). Note that none of the *spo* mutants in which the GPR processing was blocked accumulated significant DPA in the developing forespore, as found previously (4), while the *spoVI* and *gerE* mutants accumulated wild-type levels of DPA (Table 2).

Effect of GPR overexpression on sporulation, germination, and spore properties. As noted above, *B. megaterium* *gpr* expression from the *sspB* promoter on a multicopy plasmid increased spore GPR levels at least 100-fold. This elevated GPR synthesis had no apparent effect on *B. subtilis* sporulation. However, analysis of the SASP level in spores with high levels of *B. megaterium* GPR (strain PS832 with plasmid pPS1669) showed that while SASP- α levels were only slightly decreased (~30%), levels of SASP- β and SASP- γ were decreased three to fivefold (data not shown). The decreased SASP level in this strain was not due to competition for σ^G containing RNA polymerase between the *sspB* promoter driving the *gpr* gene and the strain's endogenous *sspA*, -B, and -E genes (which code for SASP- α , - β , and - γ , respectively), as expression of β -galactosidase in a strain carrying an *sspE-lacZ* fusion was not affected by plasmid pPS1669 (data not shown). This suggests that the decreased SASP level in spores with plasmid pPS1669 is due to significant SASP degradation in the developing spore. Presumably this degradation was catalyzed by the high level of P₄₁, which has full enzyme activity when assayed in vitro (7). In contrast, spores with *B. megaterium* GPR expressed at normal levels (i.e., strain PS832 with plasmid pPS1673) had normal levels of all three major SASP (data not shown).

Spores with elevated levels of either *B. subtilis* or *B. megaterium* GPR germinated and appeared to go through outgrowth and resume vegetative growth normally. In addition, transformation of the *B. subtilis* *gpr* mutant strain PS1029 with either plasmid pPS1669 or pPS1671 resulted in spores in which SASP degradation during spore germination appeared normal, and the slow spore outgrowth phenotype of the *gpr* knockout mutant (18) was suppressed (data not shown). This is further evidence that the slow outgrowth phenotype of *gpr* mutant spores is due to the slow degradation of SASP during spore germination.

TABLE 2. Synthesis and processing of GPR during sporulation of various *B. subtilis* strains^a

Relevant genotype of <i>B. subtilis</i> strain (% DPA) ^b	GPR synthesis (S) and processing (P) ^c							
	<i>B. megaterium</i>				<i>B. subtilis</i>			
	Overexpressed		Normal level		Overexpressed		Normal level	
	S	P	S	P	S	P	S	P
<i>spo</i> ⁺ (100)	++	++	++	++	++	++	++	++
<i>spoIIAC</i> (<3)	-	-	-	-	-	-	-	-
<i>spoIIIC</i> (<3)	++	-	+++	-	++	-	++	-
<i>spoIIIG</i> (<3)	-	-	+++	-	-	-	++	-
<i>spoIV</i> and <i>spoV</i> mutants (<3) ^d	++	-	++	-	++	-	++	-
<i>spoVIA</i> (95)	++	++	++	++	++	++	++	++
<i>gerE</i> (102)	++	++	++	++	++	++	++	++
<i>ΔsspA sspB</i> (97)	++	++	++	++	++	++	++	++

^a The strains of *B. subtilis* analyzed had the noted genotype and carried no plasmid (to give normal level of expression of *B. subtilis* GPR), plasmid pPS1669 (to give overexpression of *B. megaterium* GPR), plasmid pPS1673 (to give a normal level of expression of *B. megaterium* GPR), or plasmid pPS1671 (to give overexpression of *B. subtilis* GPR). The resulting strains were sporulated, samples were isolated at *t*₁₂ of sporulation, and GPR was examined by Western blot analysis as described in Materials and Methods. Invariably, the same results were obtained when samples isolated at *t*₇ were analyzed.

^b Values in parentheses are the percentages of the amount of DPA accumulated in *t*₁₂ samples relative to the level in a *spo*⁺ culture, which was set at 100%.

^c The extents of GPR synthesis and processing in untransformed cells or cells transformed with each of the three plasmids have been normalized to the levels seen in the *spo*⁺ strain at *t*₁₂ of sporulation. The latter levels have been set at ++, -, 10% or less of the *spo*⁺ level of synthesis or processing; +++, ≥150% of the *spo*⁺ level of synthesis.

^d *spo* mutants tested were *spoIVA*, *spoIVB*, *spoIVF*, *spoIVG*, *spoVA*, *spoVD*, *spoVE*, and *spoVF*.

GPR processing during germination. Analysis of GPR in dormant spores showed the presence of a significant amount of antigen which migrated at the size of the protein initially synthesized in sporulation (P₄₆), although the majority of the GPR was the smaller processed form (P₄₁) (Fig. 1, 2, and 5A to D). This was found previously in *B. megaterium* spores (11). Examination of the fate of these proteins during germination of *B. subtilis* spores with normal levels of GPR showed that *B. megaterium* P₄₁ was converted to a smaller species (P₄₀) beginning early in germination, and both P₄₀ and P₄₁ disappeared as germination continued (Fig. 5A). However, the P₄₆ initially present in the dormant spore was not significantly altered in size or amount throughout germination (Fig. 5A). These findings are similar to those made previously with *B. megaterium* GPR in *B. megaterium* spores (11). With spores containing normal levels of *B. subtilis* GPR, P₄₁ also disappeared as germination proceeded (Fig. 5B). However, the amount of sample that needed to be applied to this gel made resolution of P₄₁ and P₄₀ difficult; this particular batch of spores also had very low levels of *B. subtilis* P₄₆ (Fig. 5B). During germination of spores with elevated GPR levels, both *B. megaterium* and *B. subtilis* P₄₁ were converted to P₄₀ beginning early in germination (Fig. 5C and D). However, the P₄₁→P₄₀ conversion was not complete in these spores with elevated levels of P₄₁ (Fig. 5C and D). The level of P₄₁ plus P₄₀ decreased significantly throughout germination, but had not disappeared completely by the last time point analyzed (Fig. 5C and D). However, there was no significant change in the level of P₄₆ during germination of these spores, as noted above for spores with normal GPR levels (Fig. 5C and D). Interestingly, some of the overexpressed *B. megaterium* P₄₁ appeared to be converted to a form significantly smaller than P₄₀ late in spore germination (Fig. 5C, arrow labeled a). Possibly, this species is an intermediate in the degradation of P₄₀ or P₄₁ which occurs perhaps because some part of the normal degradation pathway has been overwhelmed by the amount of substrate.

Determination of the amino termini of various forms of GPR. To determine the differences between the various forms of GPR, dormant or germinated spores of *B. subtilis*

overexpressing either *B. megaterium* or *B. subtilis* GPR were extracted, and the proteins were run on an SDS-polyacrylamide gel and transferred to Immobilon paper. Because of the amount of GPR in these spores, it was possible to cut out the bands corresponding to P₄₆ and P₄₁ from the dormant spore extract, and the band corresponding to P₄₀ from the germinated spore extract, and subject these bands to automated protein sequence analysis. This work established that the form in which both *B. megaterium* and *B. subtilis* GPR is synthesized (P₄₆) has an amino-terminal sequence identical to that predicted from the gene's sequences (Fig. 6). In contrast, the processed form generated in sporulation (P₄₁) lacked 15 (*B. megaterium*) or 16 (*B. subtilis*) amino-terminal residues (Fig. 6). The new form generated early in spore germination (P₄₀) lacked only a single additional amino-terminal residue, a leucine in both *B. megaterium* and *B. subtilis* GPR (Fig. 6). The amino-terminal sequence of the *B. megaterium* P₄₀ isolated from *B. subtilis* spores is identical to that determined previously for P₄₀ purified from *B. megaterium* spores (27).

DISCUSSION

Analysis of *B. megaterium* GPR processing during sporulation and germination in *B. subtilis* has shown that the processing reactions, at least in terms of the size of the processed polypeptides and the amino-terminal sequence of the P₄₀ formed, are identical to those found previously in *B. megaterium* (11, 27). The *B. subtilis* GPR undergoes similar processing reactions, although the apparent sizes of the various forms of *B. subtilis* GPR differ from those of *B. megaterium*. This difference in the sizes of the forms of GPR from the two species appears due to sequence differences between the two primary gene products which have only 68% identity, with two additional residues in the *B. megaterium* GPR (27). While the *B. megaterium* P₄₆ has an apparent molecular weight of 46,000 on SDS-polyacrylamide gels, the molecular weight predicted from the gene's coding sequence is only 40,600, very similar to the value predicted for *B. subtilis* P₄₆ (40,200 [27]). Thus, *B. megaterium* P₄₆

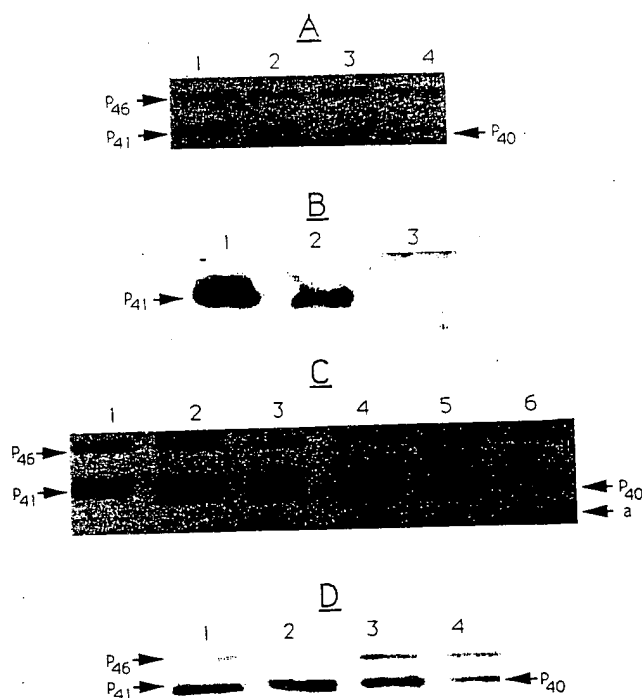


FIG. 5. Fate of *B. megaterium* or *B. subtilis* GPR during *B. subtilis* spore germination. Spores of strain PS832 carrying (A) plasmid pPS1673 (normal level of *B. megaterium* GPR), (B) with no plasmid (normal level of *B. subtilis* GPR), (C) plasmid pPS1669 (overexpressed *B. megaterium* GPR), or (D) plasmid pPS1671 (overexpressed *B. subtilis* GPR) were germinated as described in Materials and Methods, and spores were isolated, disrupted, extracted, and subjected to Western blot analysis by using anti-*B. megaterium* P₄₀ with colorimetric detection (A and C) or anti-*B. megaterium* P₄₆ with chemiluminescence detection (B and D). Labeled arrows denote the positions of P₄₆, P₄₁, and P₄₀. The germination times at which samples run in various lanes were extracted are as follows: (A) lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; and lane 4, 60 min; (B) lane 1, 0 min; lane 2, 15 min; and lane 3, 60 min; (C) lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 60 min; lane 5, 120 min; and lane 6, 200 min; (D) lane 1, 0 min; lane 2, 15 min; lane 3, 60 min; and lane 4, 90 min. The positions of P₄₆, P₄₁, and P₄₀ are noted by the horizontal arrows. In panel C, the arrow labeled a denotes a possible intermediate in *B. megaterium* GPR degradation.

may run anomalously slowly on SDS-polyacrylamide gel electrophoresis.

The only GPR processing reaction we have identified to date is loss of residues at the amino terminus. For the processing of P₄₆→P₄₁, this seems likely to be due to an endoproteolytic cleavage, although we have not shown this directly, i.e., by isolation of the peptide removed. However, the further amino-terminal processing during spore germination is clearly exoproteolytic, as only a single amino-terminal residue is removed in the P₄₁→P₄₀ conversion. It is possible, of course, that there are additional carboxyl-terminal proteolytic processing reactions, and the apparent loss of ~5,000 in the molecular weight of *B. megaterium* GPR going from P₄₆ to P₄₁ would be consistent with this idea. While we have not yet addressed this point experimentally, the *B. subtilis* GPR loses only ~2 kDa in conversion of P₄₆ to P₄₁, consistent with removal of only 16 amino-terminal residues. As noted above, it seems likely that the migration differences between the various forms of GPR from *B. megaterium* and

B. subtilis are due to differences in primary sequence between the proteins from the two species.

Whatever the reactions involved in GPR processing, the enzymes catalyzing these reactions appear capable of processing much more GPR than is normally present in a wild-type organism. One explanation for this observation is that one or both GPR processing steps are self-catalyzed, and thus GPR overexpression would increase both the substrate and the enzyme involved in the processing. While self-processing may well be the mechanism of P₄₆→P₄₁ conversion (see below), the P₄₁→P₄₀ conversion is almost certainly catalyzed by an enzyme distinct from GPR (7). Clearly, the latter enzyme must be present in a significant excess over the amount needed to process normal substrate levels. However, the very slow conversion of P₄₁→P₄₀ during germination of spores with overexpressed *B. megaterium* GPR may indicate that the catalytic capacity of the enzyme catalyzing the P₄₁→P₄₀ conversion may have been reached. Our finding that the P₄₁→P₄₀ conversion involves removal of only a single amino-terminal residue suggests that an aminopeptidase catalyzes this processing reaction. Spores contain high levels of an aminopeptidase which is very active in removal of hydrophobic residues (20); possibly, this is the enzyme catalyzing the germination-specific processing. Previous work with *B. megaterium* spores has shown that both P₄₁ and P₄₀ are lost completely during spore germination and appear to be degraded by ATP-dependent proteolysis (11). *B. subtilis* spores with normal GPR levels also lose both P₄₁ and P₄₀ during germination. The retention of significant amounts of these protein species during germination of spores with overexpressed GPR suggests that the elevated levels of P₄₁ and/or P₄₀ may have overloaded the proteolytic system which normally catalyzes their degradation. However, this overload had no apparent effect on development of these spores, as they appeared to resume vegetative growth normally.

Probably the major unanswered question about GPR processing concerns the identity of the enzyme catalyzing the P₄₆→P₄₁ conversion. As found previously in experiments examining processing of endogenous *B. megaterium* P₄₆ in *B. megaterium* forespore extracts (7), we too have not observed appropriate processing of *B. megaterium* P₄₆ by extracts from *B. subtilis* forespores isolated 5 to 7 h into sporulation (19). However, as suggested previously (7), this processing reaction takes place while the forespore is undergoing dramatic physiological changes, i.e., significant dehydration. Thus, the P₄₆→P₄₁ conversion may require conditions very different from those commonly used for in vitro reactions. Indeed, P₄₆ not converted to P₄₁ during sporulation is not processed during spore germination. While it is certainly possible that there is a separate processing enzyme that catalyzes the P₄₆→P₄₁ conversion, we think it is more likely that this process is self-catalyzed. By self-catalyzed we do not mean autocatalytic, as it is clear that neither P₄₀ nor P₄₁ can process P₄₆ under normal cellular conditions (7, 19). Rather, we suggest that each molecule of P₄₆ processes itself, and only itself, to P₄₁ by proteolysis. There are, we think, three pieces of evidence for this proposal. First, 100-fold higher than normal levels of GPR are processed to approximately the same extent as normal levels; i.e., only 60 to 90% of P₄₆ is converted to P₄₁ during sporulation. If this processing reaction was a cleavage catalyzed by a distinct GPR-specific protease present in limiting amounts (hence, not all of the P₄₆ being processed), one might have expected that this processing enzyme would have been overwhelmed by GPR overexpression. However, the extent and timing of

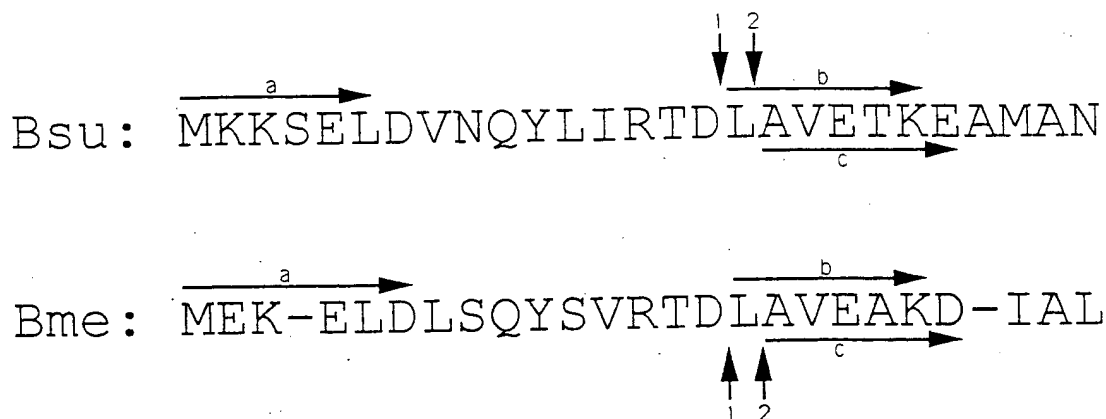


FIG. 6. Amino acid sequences at the amino termini of various forms of *B. megaterium* and *B. subtilis* GPR. The amino acid sequences of the *B. subtilis* (Bsu) or *B. megaterium* (Bme) GPR are given in the one-letter code; they were taken from the sequence predicted from the genes' coding sequences (27) and begin at the initiating methionine residue. The horizontal arrows above and below the sequences and which are labeled with small letters give the results of six cycles of automated sequenator analysis of the following: a, the band corresponding to P_{46} in dormant spores with overexpressed GPR; b, the band corresponding to P_{41} in dormant spores with overexpressed GPR; and c, the band corresponding to P_{40} in 30-min-germinated spores with overexpressed GPR. For each sequencing cycle the residue noted was by far the predominant residue detected (4 to 10 times higher than any other amino acid). The vertical arrows labeled 1 and 2 denote the sites of the GPR processing steps during conversion to P_{41} and P_{40} , respectively.

the processing of the overexpressed P_{46} were essentially the same as when normal levels of GPR were produced. Second, a mutant form of *B. megaterium* GPR with little or no catalytic activity in vivo exhibits greatly decreased $P_{46} \rightarrow P_{41}$ processing during sporulation (11). While there are certainly alternative explanations for the latter finding, isolation and analysis of an inactive GPR by mutation of the putative active-site serine residue (10) would be a good test of the idea of self-processing. Third, and we think, the strongest evidence comes from comparison of the sequence around the $P_{46} \rightarrow P_{41}$ cleavage sites in *B. megaterium* and *B. subtilis* GPR with the sequences in sites recognized and cleaved in SASP by P_{40} or P_{41} (Fig. 7). Strikingly, the conserved

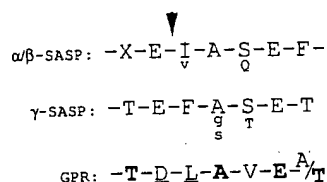


FIG. 7. Comparison of the amino acid sequences around GPR cleavage sites in SASP with the sequence in the $P_{46} \rightarrow P_{41}$ cleavage site. Amino acid sequences are given in the one-letter code. The SASP cleavage sites are taken from reference 21 and include information only from *B. cereus*, *B. megaterium*, and *B. subtilis* SASP. The vertical arrow denotes the bond cleaved in the three types of proteins. The residues shown are those from 6 cleavage sites in γ -type SASP from these species (γ -SASP), as well as 13 cleavage sites in α/β -type SASP (α/β -SASP). Single residues shown in the sequences around cleavage sites in SASP are the only residues found at this position; for positions with variation but only one variant residue, the minor residue(s) is given in lowercase letters below the predominant residue. The small capital letters below the major residue denote the residue found at this position in four (Q) or two (T) cleavage sites. The letter X as the first residue of the sequence around the α/β -SASP cleavage site notes that there is no consensus residue at this position. Residues in the $P_{46} \rightarrow P_{41}$ cleavage site which are identical to those in GPR cleavage sites in γ -type SASP are in boldface; type residues which are similar to those in GPR cleavage sites in SASP are underlined.

heptapeptide surrounding the $P_{46} \rightarrow P_{41}$ cleavage sites shares three or four identical residues with the sites cleaved in γ -type SASP of *B. cereus*, *B. megaterium*, and *B. subtilis*. Of the three other residues, two are very similar to residues in SASP cleavage sites, with only one which is very different (Fig. 7). Given the high sequence specificity of GPR (21), this sequence similarity strongly suggests that GPR cleaves itself at a site similar to that in its SASP substrates. One possible model is that the amino-terminal peptide in P_{46} actually sits in the enzyme's active site, inhibiting enzyme activity; initially, the Asp-Leu bond does not align properly with the enzyme's catalytic serine residue and is therefore not cleaved. However, as sporulation proceeds, there is a conformational change in P_{46} , possibly associated with spore core dehydration (see below), which then allows cleavage of the Asp-Leu bond converting P_{46} to P_{41} . If the change in P_{46} stimulating processing to P_{41} is indeed the result of removal of significant water from the spore core, then the low water level when P_{41} is generated might be the reason for the low activity of P_{41} on its SASP substrates in vivo at this time, i.e., because of restrictions in protein diffusion. Similarly, the spore's aminopeptidase would be unable to process P_{41} to P_{40} at this time. We have found that the processing of GPR during sporulation takes place at or slightly before the uptake of the large depot of DPA (up to 10% of the spore's dry weight) by the forespore, which itself takes place as the spore core becomes dehydrated, resulting in spore heat resistance (5). It would have been most informative to have been able to elevate the DPA level in forespores of the *spoVF* mutant, which is thought to lack DPA synthetase (4), by exogenous DPA. Unfortunately, while sporulation of this strain with exogenous DPA results in a significant titer of heat-resistant spores, this titer remains ~ 2 logs below that of a *spo+* culture (4, 19), and the amount of DPA taken up by the culture as a whole is insignificant.

In addition to GPR processing, there are at least two other proteolytic processing reactions which serve key regulatory functions in sporulation. Both are the proteolytic activation of precursor forms of sporulation-specific sigma factors for RNA polymerase, pro- σ^E and pro- σ^K , to their active forms

(12). The amino acid sequences in the regions around the cleavage sites in these two pro- σ factors bear no resemblance to that around the cleavage sites in GPR (8, 13). However, for neither of the pro- σ factors has any correct in vitro processing been achieved. Indeed, it has been suggested that these processing reactions, in particular, that of pro- σ^E , may be coupled to a morphological change in the sporulating cell (3, 23). In some respects this proposal is analogous to our proposal that P_{46} to P_{41} processing is triggered by a profound physiological change in the developing forespore.

A final question to be considered is the function of each of the GPR processing reactions. Previous work has shown that *B. megaterium* P_{46} is inactive both in vivo and in vitro; in contrast, P_{41} is active in vitro, but must have little or no activity in vivo because SASP normally do not turn over during sporulation (7, 11, 21). The fact that more than 100-fold overexpression of GPR still allows significant SASP accumulation in spores is consistent with P_{41} having very low activity inside the developing forespore. However, the decreased SASP level in these spores argues that P_{41} can have some, albeit low or transient, activity in vivo. Thus, the function of the sporulation processing of GPR appears to be to generate an enzyme which will be active in the first minutes of germination, yet have little if any activity in the developing forespore and dormant spore. This suggests that the same conditions that allow the $P_{46} \rightarrow P_{41}$ conversion during sporulation, possibly the onset of spore core dehydration as noted above, may also preclude P_{41} from diffusing to and acting on its SASP substrates. Possibly some knowledge of the conditions giving $P_{46} \rightarrow P_{41}$ processing in vitro will suggest mechanisms whereby the P_{41} generated will act poorly, if at all, on its SASP substrates in vivo.

The precise function of the germination-specific processing is even less clear, as it removes only a single amino acid, and P_{40} appears indistinguishable catalytically from P_{41} (7). One possible explanation is that the P_{41} to P_{40} processing is irrelevant to the activity of GPR in vivo during spore germination and takes place only because of the high aminopeptidase activity in the spore which presumably can only act on P_{41} in the first minutes of germination. This explanation is consistent with the lack of any clear catalytic difference between P_{41} and P_{40} in vitro (7). A second possible explanation is that removal of the amino-terminal leucine renders the resulting P_{40} susceptible to the cell's ATP-dependent proteolytic system which degrades P_{40} as spore outgrowth proceeds (11). There is good precedent for such processes in *E. coli*, yeasts, and higher organisms (1, 28). However, in the latter organisms generation of an amino-terminal alanine residue would not result in protein destabilization, as alanine is generally an amino-terminal stabilizing residue (1, 28). While the identities of stabilizing and destabilizing amino-terminal residues have not been established in *Bacillus* species, the relative similarity of the behavior of various residues in both *E. coli* and yeasts suggests that this would also be similar in *B. subtilis*. Consequently, at present the reason for the P_{41} to P_{40} processing is unclear.

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COMMENTS

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Initiation of the Germination of *Bacillus subtilis* Spores by a Combination of Compounds in Place of L-Alanine

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L-Alanine initiates the germination of spores of *Bacillus subtilis* by entering two metabolic pathways. The products of one pathway, which is inhibited by D-alanine or by elevated temperature, can also be derived from a combination of fructose, glucose, and K⁺. The present study demonstrated that the products of the other pathway can be derived from L-asparagine or L-glutamine or, to a lesser extent, from several other amino acids. Hence, the combination of L-asparagine (or L-glutamine), fructose, glucose, and K⁺ can initiate spore germination in the absence of L-alanine. Spores preincubated in a combination of asparagine and fructose do not lose refractility, optical density, or heat resistance, and do not take up methylene blue stain. The spores do, however, undergo some reaction which prepares them for a more rapid response to the later addition of glucose and K⁺. This preincubation reaction has an optimal temperature of about 44 C.

The germinative process of typical *Bacillus subtilis* spores can be initiated by 10⁻³ M L-alanine (ALA) or by any one of certain other amino acids. The rate of germination can be measured by the decrease in optical density (OD), at 625 m μ , of a spore suspension in tris(hydroxymethyl)aminomethane (Tris) buffer. Previous experiments (7) indicated that, in the germinative process, ALA enters two metabolic pathways. One pathway is inhibited by D-alanine or by high temperatures (49 C). The products of this pathway, which are necessary for initiation, can also be derived from a combination of fructose (FRU), glucose (GLC), and K⁺. It seemed possible that the product(s) of the other pathway of ALA also could be derived from a combination of compounds that are incapable of initiating spore germination by themselves. We found that, in the presence of FRU, GLC, and K⁺, germination was also initiated by the addition to a spore suspension of L-asparagine (ASN), L-glutamine (GLN), or, to a lesser extent, L-cysteine, L-serine, or glycine.

MATERIALS AND METHODS

Strains. The transformable strain 60127 (nicotinic acid⁻) was used for studies on *B. subtilis*. *B. cereus* strain T was obtained from B. Krask.

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TBAB plates. TBAB plates contained 33.0 g per liter of Tryptose Blood Agar Base (Difco).

Sporulation, harvest, heat activation, and initiation procedures. These procedures were described previously (7). Except where indicated, the initiating agents were used at the following final concentrations: KCl, 3.3 mg/ml; ALA, 0.1 mg/ml; ASN, 0.33 mg/ml; GLC, 1 mg/ml; FRU, 1 mg/ml; and 0.1 M Tris-chloride buffer.

The rate of initiation was measured by k_m , the maximal value of the rate at which the function OD/OD₀ decreased per hr (OD measured at 625 m μ ; OD₀ = initial optical density of the spore suspension at 625 m μ).

Stainability. Stainability was determined by adding a drop of 0.5% methylene blue to dried spores on a slide, placing a glass cover slip on the slide, and examining the spores in a light microscope 5 min later.

Tween 80 was purchased from the Atlas Powder Co. (Wilmington, Del.).

RESULTS

Figure 1 shows the initiation of 60127 spores by ASN or GLN in the presence of FRU plus GLC plus K⁺. When any of these compounds was left out, no initiation was observed (some spore preparations showed a slow rate of initiation without added potassium, presumably because traces of potassium were still present). ALA alone initiated germination of spores at a slightly higher rate than the above combinations. The initiation rates (k_m) observed with other amino

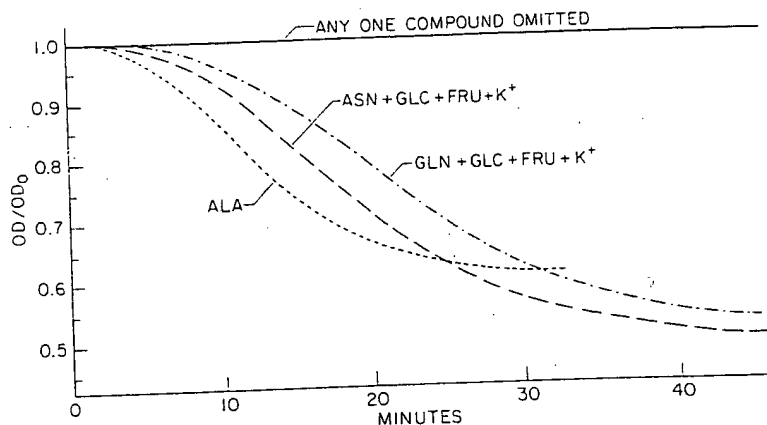


FIG. 1. Initiation of spores by ALA and by the combinations of ASN + FRU + GLC + K⁺ and GLN + FRU + GLC + K⁺. All reactions were run at 37 C in 0.1 M Tris-chloride, pH 7.4.

acids, alone and in the presence of FRU plus GLC plus K⁺, are summarized in Table 1. The effects of all amino acids which could initiate germination alone were potentiated by the addition of FRU plus GLC plus K⁺. The addition of these substances was especially effective for L-cysteine, glycine, and L-serine.

Table 2 shows that glucosamine could replace GLC, and mannose could replace FRU, but five times higher concentrations of these compounds were required to obtain comparable values of the maximal initiation rate, k_m . Many other carbon sources (at 1 mg/ml) were ineffective as replacements for either GLC or FRU (see Table 2).

The temperature dependence of the initiation rates (k_m) is shown in Fig. 2. Although a combination of ALA, FRU, GLC, and K⁺ initiated germination at a higher rate than a combination of ASN, FRU, GLC, and K⁺, the temperature optima differed only slightly. Heat-shocked spores reacted more than twice as fast as nontreated spores.

Figure 3 shows how the initiation rates (k_m) varied with the concentration of one of the four compounds (ASN, FRU, GLC, K⁺) when the other three were in excess. It is apparent that FRU could not replace GLC and vice versa, even at high concentrations (1 mg/ml).

Although the refractility of the spores did not change when they were exposed to fewer than the above four compounds, some biochemical reaction may have taken place. Therefore, spore suspensions were exposed for 1 hr at 37 C to different combinations of ASN, FRU, GLC, and K⁺; subsequently, the compound(s) left out was added. Throughout this experiment, the change in OD was recorded. When both ASN and FRU were initially present, a much more rapid initiation was observed upon subsequent addition of

GLC and K⁺ than when all four compounds were added simultaneously. The early addition of a mixture ASN, GLC, and K⁺ or of a mixture of FRU, GLC, and K⁺ did not cause such an effect (Fig. 4). The presence of K⁺, in addition to ASN and FRU, did not influence the subsequent response to GLC and K⁺. It is therefore clear that the reaction involving GLC, but not the reaction involving ASN or FRU, required K⁺.

During the period of exposure to ASN and FRU, spores did not become stainable by methylene blue, did not lose their refractility, and did not become heat-sensitive (survival was measured, after heating for 30 min at 78 C, by plating on TBAB). The spores did, however, tend to form clumps and to become attached to glass or plastic centrifuge tubes. At the suggestion of A. Keynan, Tween 80 (0.33 mg/ml) was used to avoid the stickiness and thus facilitate medium changes after the preincubation period. The concentration of Tween 80 used had no effect on initiation rates in a mixture of ASN, FRU, GLC, and K⁺. When spores were preincubated for 1 hr at 37 C in ASN plus FRU, then were centrifuged in the cold for 4 min at 9,000 rev/min (9,700 × g), and were finally resuspended in Tris plus Tween 80, the addition of GLC plus K⁺ effected only a small OD decrease. When all four compounds were added after centrifugation, however, the usual rapid OD decrease was observed. This result indicates that ASN plus FRU must be present at the same time as GLC and K⁺ to allow initiation to continue.

The optimal temperature for the preincubation reaction was measured by suspending heat-activated spores in a solution of 0.1 M Tris-chloride (pH 7.4) plus ASN (0.33 mg/ml) plus FRU (1 mg/ml) at different temperatures. At different times, samples were removed and were

TABLE 1. Initiation rates (k_m) observed for different nitrogen sources^a

Compound	Compound alone			In the presence of FRU + GLC + K ⁺		
	333 μ g/ml	100 μ g/ml	33 μ g/ml	333 μ g/ml	100 μ g/ml	33 μ g/ml
Adenosine.....				<0.01		
β -Alanine.....	0.09			0.2	0.03	0.03
L-Alanine.....	1.7	1.7	1.4	2.1	2.1	2.1
L- α -Aminobutyrate.....	1.1	0.86	0.44	1.7	1.1	0.66
L- γ -Aminobutyrate.....	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
L- α -Aminoisobutyrate.....	0.98	0.59	0.17	1.2	0.56	0.23
L-Arginine.....				<0.01		
L-Asparagine.....	<0.01			1.3	1.1	0.09
L-Aspartate.....				0		
L-Cysteine.....	<0.01			0.54	0.26	<0.01
D-Galactosamine.....				<0.01		
D-Glucosamine.....				<0.01		
L-Glutamate.....				<0.01		
L-Glutamine.....	<0.01			1.2	0.38	<0.01
Glycine.....	<0.01			0.50	0.24	<0.01
L-Histidine.....				<0.01		
Inosine.....	0.08			<0.01		
L-Isoleucine.....				0.20	0.05	0.01
L-Leucine.....				<0.01		
L-Lysine.....				<0.01		
L-Methionine.....				<0.01		
L-Norvaline.....	0.33			0.66	0.14	0.02
L-Phenylalanine.....				0		
L-Proline.....				0		
L-Serine.....	<0.01			0.19		
L-Threonine.....				<0.01		
L-Tryptophan.....				<0.01		
L-Tyrosine.....				<0.01		
L-Valine.....	0.29			0.54	0.06	0.03

^a FRU and GLC were used at a concentration of 1 mg/ml, whereas KCl was used at a concentration of 3.3 mg/ml.

TABLE 2. Initiation rates (k_m) in the presence of ASN (0.330 mg/ml) \pm KCl (3.3 mg/ml) + 0.1 M Tris-chloride, pH 7.4^a

Combinations of initiating agents	K_m
GLC (1 mg/ml) + mannose (0.33 mg/ml).....	1.4
GLC (1 mg/ml) + mannose (0.10 mg/ml).....	1.3
GLC (1 mg/ml) + mannose (0.03 mg/ml).....	0.74
FRU (1 mg/ml) + glucosamine (1.0 mg/ml).....	1.3
FRU (1 mg/ml) + glucosamine (0.1 mg/ml).....	0.6

^a The following compounds (at 1 mg/ml) showed $k_m < 0.01$ when they replaced either GLC or FRU: *n*-acetyl glucosamine, adenosine, L-arabinose, fructose-6-phosphate, fructose-1,6-diphosphate, D-fucose, D-galactitol, D-galactose, L-glucose, glucose-6-phosphate, DL-glyceraldehyde, glycerol, inosine, L-inositol, lactose, levo-glucosan, D-lyxose, levulinic acid, β -methyl-D-glucoside, pyruvate, rhamnose, ribose, sorbose, sucrose, and D-xylose.

adjusted to 37 C; initiation was started by the addition of GLC (1 mg/ml) and K⁺. The initiation rates increased with the time of preincubation until a maximal k_m was obtained (see Fig. 5). The k_m values obtained after 1 hr of preincubation were plotted against temperature (Fig. 6). The optimal temperature for the preincubation reaction was approximately the same as the optimal temperature for the overall initiation reaction.

The maximal k_m values obtained after preincubation depended on the temperature employed (Fig. 5). This finding suggested the presence of an equilibrium between production and elimination from spores of a compound needed for initiation, the equilibrium constant depending on the temperature. To test this possibility, spores were incubated in ASN plus FRU for 1 hr at 42.5 C (a temperature giving the maximal rate of subsequent initiation at 37 C). The spores were then kept in ASN plus FRU at 0 or at 28 C for 7 hr before they were exposed to

C + K⁺ and GLN + FRU

all four compounds were. The early addition of a d K⁺ or of a mixture of l not cause such an effect. K⁺, in addition to ASN, influence the subsequent re- It is therefore clear that LC, but not the reaction required K⁺.

f exposure to ASN and some stainable by methy- their refractility, and did ive (survival was meas- 0 min at 78 C, by plating id, however, tend to form attached to glass or plastic suggestion of A. Keynan,) was used to avoid the ciliate medium changes period. The concentration o effect on initiation rates U, GLC, and K⁺. When ed for 1 hr at 37 C in re centrifuged in the cold in (9,700 \times g), and were Tris plus Tween 80, the K⁺ effected only a small l four compounds were ion, however, the usual observed. This result indi- U must be present at the K⁺ to allow initiation to

ure for the preincubation d by suspending heat- solution of 0.1 M Tris- A⁺ 0.33 mg/ml) plus lferent temperatures. At were removed and were

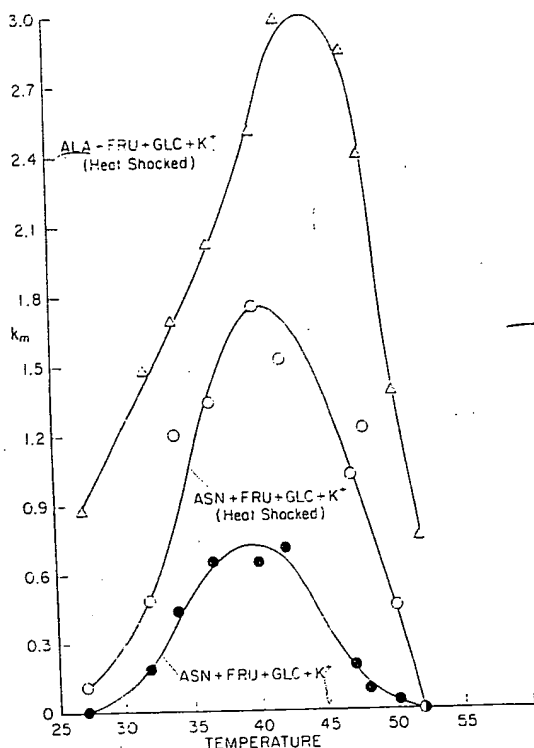


FIG. 2. Dependence of initiation rates (k_m) on temperature and heat shock in the presence of ALA + FRU + GLC + K^+ or ASN + FRU + GLC + K^+ . All samples were run in 0.1 M Tris chloride, pH 7.4.

GLC plus K^+ at 37°C. Although spores which were exposed to GLC plus K^+ immediately after preincubation showed a k_m of 5.4, those spores which were kept for 7 hr at 0 or at 28°C showed k_m values of 5.4 and 2.6, respectively.

D-Alanine did not inhibit the initiation induced by a combination of ASN, FRU, GLC, and K^+ , even when the ratio of D-alanine to ASN was 10:1. In contrast, a D-alanine to ALA ratio of 10:1 was sufficient to prevent initiation by a mixture of ALA, FRU, GLC, and K^+ (7).

After heat treatment, *B. cereus* (strain T) can be initiated by 100 μ g/ml of ALA if hydroxylamine (0.01 M) is present (B. Krask, personal communication). Such spores did not show any initiation in a mixture of ASN, FRU, GLC, and K^+ , whether or not hydroxylamine was present. A partial initiation (Table 3), however, was obtained in a mixture of ALA, ASN, FRU, and GLC in the absence of hydroxylamine.

DISCUSSION

Several amino acids, especially asparagine and glutamine, which by themselves cannot initiate

TABLE 3. Initiation of *Bacillus cereus* strain T spores by combinations of ASN (0.33 mg/ml), ALA (0.1 mg/ml), 0.1 M hydroxylamine (HA), GLC (1 mg/ml), and FRU (1 mg/ml) in 0.1 M Tris-chloride, pH 7.4

Heat-shocked ^a	Combinations of initiating agents					k_m	OD ₆₉₀ /OD ₇₃₀
	HA	ASN	GLC	FRU	ALA		
+	-	+	+	+	-	0.01	0.98
+	-	+	+	+	+	3.3	0.65
+	+	-	-	-	+	5.9	0.42
+	-	-	-	-	+	<0.01	1.0
+	-	-	+	+	+	<0.01	1.0
+	-	+	+	+	+	<0.01	1.0
+	-	+	-	+	+	<0.01	1.0
+	-	+	-	-	+	<0.01	1.0
+	+	+	+	+	-	<0.01	1.0
+	+	+	+	+	+	<0.01	1.0
+	+	-	-	-	+	<0.01	1.0
+	-	+	+	+	+	<0.01	1.0

^a Where indicated, spores were heat shocked in water for 1 hr at 70°C.

the germination of *B. subtilis*, can do so in the presence of FRU plus GLC plus K^+ . The initiation of *B. subtilis* spores by asparagine plus caramelized glucose was reported by Hachisuka et al. (13). The active agents in "caramel" apparently are FRU and GLU (7). The finding of several agents, which can initiate only in combination, made it possible to study the sequential action of the individual components. A reaction occurs in the presence of ASN plus FRU, which prepares the spores for a rapid initiation when GLC plus K^+ are subsequently added. In the spore, ASN and FRU apparently give rise to a metabolite which is necessary for initiation. When this compound is not utilized for initiation soon after its production, it is lost again, apparently by enzymatic breakdown rather than by simple diffusion out of the spore. An enzymatic degradation is indicated because the preincubation response for rapid initiation, obtained at 42.5°C, is lost after several hours at 28°C but is stable for many hours at 0°C. Although preincubation in ASN plus FRU enhances the subsequent response to GLC plus K^+ , all of these compounds are needed continuously to give complete initiation.

K^+ ions were not needed during preincubation in ASN plus FRU. The ions apparently are necessary for the uptake or utilization of glucose.

In a recent paper (2), it was proposed that ALA initiates germination by two metabolic pathways (Fig. 7). The pathway to compound II, and on to

of *Bacillus cereus* strain T
ons of ASN (0.33 mg/ml),
.1 M hydroxylamine (HA),
and FRU (1 mg/ml) in
chloride, pH 7.4

Ions of agents		k_m	OD ₄₈₀ /OD ₀
FRU	ALA		
+	-	0.01	0.98
+	+	3.3	0.65
-	+	5.9	0.42
-	+	<0.01	1.0
+	+	<0.01	1.0
-	+	<0.01	1.0
+	+	<0.01	1.0
-	+	<0.01	1.0
+	+	<0.01	1.0
-	+	<0.01	1.0
+	+	<0.01	1.0
-	+	<0.01	1.0
+	+	<0.01	1.0

pores were heat shocked in

sub can do so in the
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K⁺, all of these compounds
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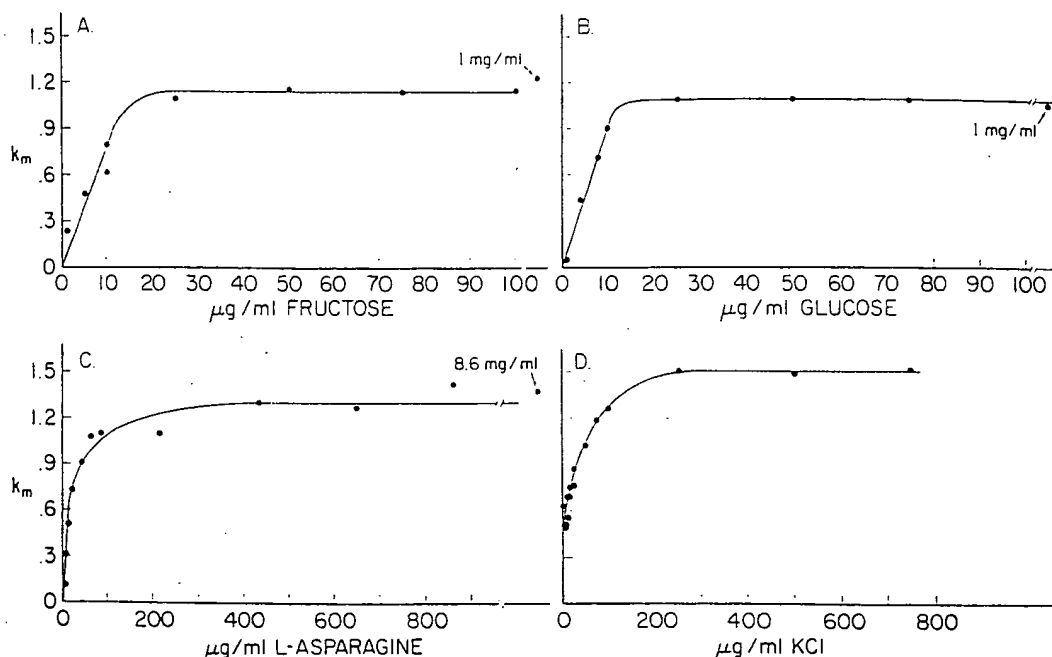


FIG. 3. Concentration dependence of initiation by ASN + FRU + GLC + K⁺ in 0.1 M Tris-chloride, pH 7.4. In each case, the concentration of one compound was varied, whereas the other three compounds were added in excess: FRU 1 mg/ml; GLC, 1 mg/ml; ASN, 0.33 mg/ml; and KCl, 3.3 mg/ml. (A) FRU varied. (B) GLC varied. (C) ASN varied. (D) K⁺ varied.

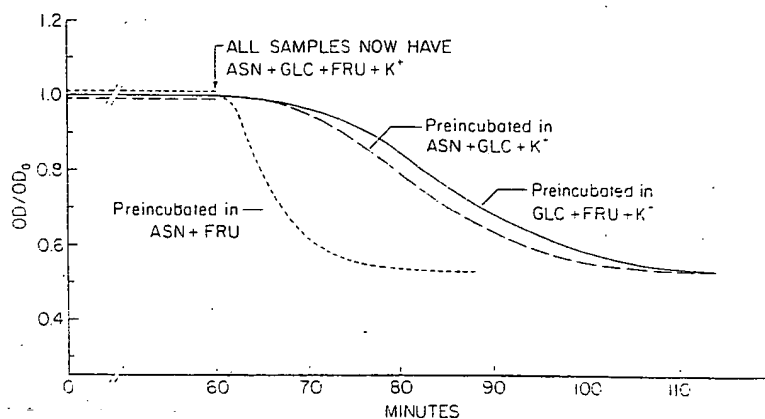


FIG. 4. Effect of preincubation at 37 C, in the presence of several compounds, on the subsequent initiation in ASN + FRU + GLC + K⁺ at 37 C.

III and IV, which is not necessary in the presence of FRU plus GLC plus K⁺, is blocked by high temperature (49 C), by D-alanine (at concentrations equal to those of ALA used for initiation), or in a mutant (7). In this pathway, ALA apparently serves as a carbon donor. In the pathway to compound I, ALA serves as an amino donor, since it can be replaced by ASN or GLN, if FRU plus GLC plus K⁺ are available. The

response of spores to preincubation in ASN plus FRU further indicates that ASN (or a product of it) and compound III (derived from FRU) must react to form compound I. Analogous to this would be the reaction of GLN or ALA with compound III to form compound I.

Our results show clearly that the initiation by ALA represents a complex metabolic process requiring the existence of many intact enzymes in

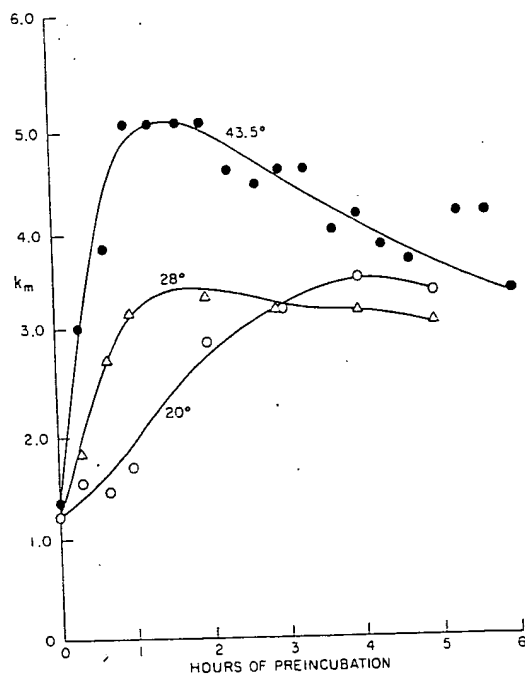


FIG. 5. Effect of preincubation time and temperature on the subsequent initiation rates at 37°C. Spores were held in a mixture of 0.1 M Tris-chloride, pH 7.4, ASN (0.33 mg/ml), and FRU (1 mg/ml), at the indicated temperatures. After different times, samples were adjusted to 37°C. GLC (1 mg/ml) + KCl (3.3 mg/ml) were then added, and the OD decrease was followed in a recording spectrophotometer.

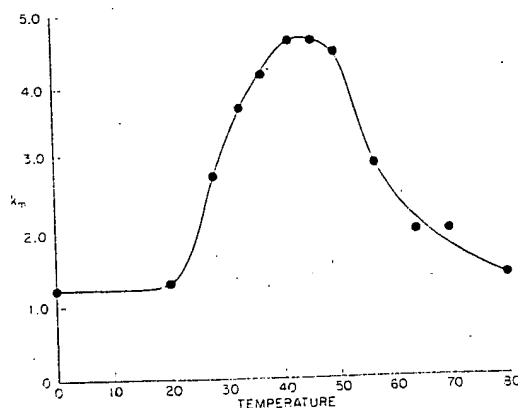


FIG. 6. Effect of temperature on the ASN + FRU preincubation reaction. Spore suspensions were incubated at various temperatures for 1 hr in ASN (0.33 mg/ml) + FRU (1 mg/ml). After transfer to 37°C, GLC (1 mg/ml) + KCl (3.3 mg/ml) were added, and initiation was followed.

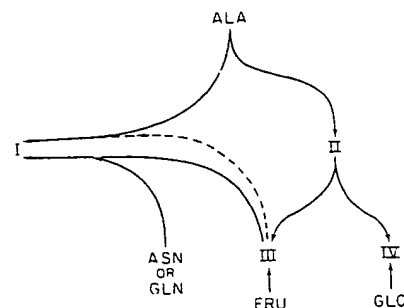


FIG. 7. Proposed scheme showing the replacement of alanine as an initiating agent by the combination of ASN + FRU + GLC or GLN + FRU + GLC. Compounds I and IV must be made to produce initiation (decrease in OD of the spore suspension). ASN, GLN, or ALA react with compound III to form compound I.

spores. The same conclusion can be derived from earlier experiments which showed that spores can be initiated by different compounds (2, 4, 6), that certain spores can be better initiated or only initiated by the combination of two agents (2, 5), or that mutants which have an additional germination requirement can be derived (1, 7).

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Comparative Study of Pressure-Induced Germination of *Bacillus subtilis* Spores at Low and High Pressures

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We have studied pressure-induced germination of *Bacillus subtilis* spores at moderate (100 MPa) and high (500 to 600 MPa) pressures. Although we found comparable germination efficiencies under both conditions by using heat sensitivity as a criterion for germination, the sensitivity of pressure-germinated spores to some other agents was found to depend on the pressure used. Spores germinated at 100 MPa were more sensitive to pressure (>200 MPa), UV light, and hydrogen peroxide than were those germinated at 600 MPa. Since small, acid-soluble proteins (SASPs) and dipicolinic acid (DPA) are known to be involved in spore resistance to UV light and hydrogen peroxide, we studied the fate of these compounds during pressure germination. DPA was released upon both low- and high-pressure germination, but SASP degradation, which normally accompanies nutrient-induced germination, occurred upon low-pressure germination but not upon high-pressure germination. These results adequately explain the UV and hydrogen peroxide resistance of spores germinated at 600 MPa. The resistance to pressure inactivation of 600-MPa-germinated spores could also, at least partly, be attributed to α/β -type SASPs, since mutants deficient in α/β -type SASPs were more sensitive to inactivation at 600 MPa. Further, germination at 100 MPa resulted in rapid ATP generation, as is the case in nutrient-induced germination, but no ATP was formed during germination at 600 MPa. These results suggest that spore germination can be initiated by low- and high-pressure treatments but is arrested at an early stage in the latter case. The implications for the use of high pressure as a preservation treatment are discussed.

A major obstacle to the application of high hydrostatic pressure as a technology for the preservation of foods and pharmaceuticals is the inefficient inactivation of bacterial spores. Ungerminated bacterial spores are believed to be extremely pressure resistant (1, 13). Spores of *B. subtilis* were shown to survive pressure treatments at 1,000 MPa for 40 min at temperatures below 10°C (17). However, it has been observed for the spores of various *Bacillus* spp. that inactivation was more efficient at moderate (200 to 500 MPa) than at higher (>500 MPa) pressure. This was explained by the finding that pressure can induce spores to germinate and lose their resistance to high pressure and heat and by the assumption that this germination is less efficient at high pressure (5, 7, 20, 26). This pressure-induced germination was strongly temperature dependent, being virtually absent at <10°C and most prominent at 40 to 50°C.

In line with these observations, it has been demonstrated that significant reductions in spore survival can be obtained by application of a cyclic process alternating between low and high pressures at moderate temperatures (40 to 70°C) (4, 10, 26). A problem that remains unsolved is the existence of a relatively large fraction of superdormant spores that remain ungerminated and thus viable after prolonged pressurization (20). There is only limited knowledge about the factors affecting pressure-induced germination and about its mechanism.

Gould and Sale (7) assumed that pressure-induced germination is caused by activation of enzymes involved in spore germination. For instance, they showed that a pressure of 25 MPa increased the activity of alanine racemase in *B. cereus* T spores. It is possible that pressure results in a changed environment in the spores by affecting the permeability of the spore envelope. The changed environment, in turn, could lead

to activation of germination enzymes. Alternatively, pressure could directly induce conformational changes in enzymes, which could lead to activation of the enzymes (16). The observation that inhibitors of nutrient-induced germination also inhibited pressure-induced germination suggests that common enzymatic reactions are involved in both germination processes (7).

The reduced germination assumed to occur at higher pressures could be explained by conformational changes leading to inactivation of a critical germination enzyme. This inactivation must be reversible, since spores exposed to 800 MPa at 25°C germinate normally at 0.1 MPa in the presence of nutrients (8).

That spore inactivation exhibits a maximum as a function of pressure has been attributed by most investigators to the existence of a pressure maximum for spore germination. However, the data reported by Gould and Sale (7) shows that at 45°C, the degree of pressure-induced germination of *B. coagulans* spores increases between 100 and 600 MPa, while the inactivation is maximal at 300 MPa and decreases at higher pressures. In this case, the pattern of spore germination cannot explain the existence of a pressure maximum for spore inactivation. In the present paper, we report a similar discrepancy between *B. subtilis* spore germination and inactivation by high pressure. In addition, we provide an explanation by demonstrating that spores germinated at high pressure pass through an incomplete germination process and therefore retain some of their resistance properties.

MATERIALS AND METHODS

Preparation of *B. subtilis* spore suspensions. *B. subtilis* ATCC 6051, obtained from the LMG culture collection (Ghent, Belgium), and *B. subtilis* PS832 (wild-type), PS356 (small, acid-soluble protein [SASP] α/β), PS483 (SASP γ), and PS482 (SASP $\alpha/\beta/\gamma$), obtained from P. Setlow (University of Connecticut Health Center), were used throughout this study. To induce sporulation, cells from a -80°C glycerol stock culture were grown at 37°C in a humid atmosphere on the surface of nutrient agar CM3 (Oxoid, Basingstoke, United Kingdom) supplemented with 0.03-g/ml $MgSO_4$ and 0.25-g/ml KH_2PO_4 . After 7 days, spores were harvested, washed two times by centrifugation at 4,000 \times g for 15 min each time, and finally resuspended in sterile, deionized water. The spore

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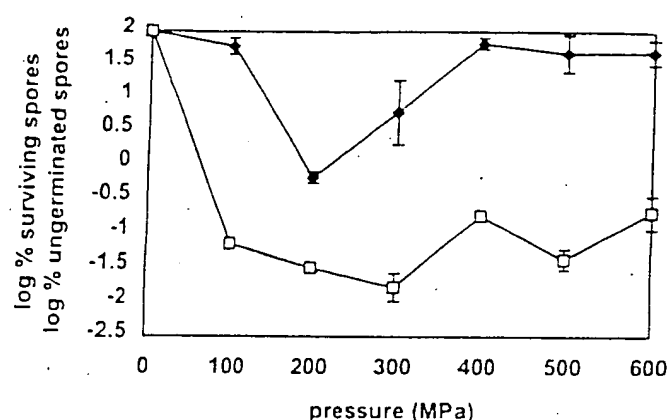


FIG. 1. Surviving spores (♦) and ungerminated spores (□) of *B. subtilis* after treatment at different pressures for 30 min at 40°C.

suspension was adjusted to 10^7 to 10^8 spores ml⁻¹ and kept at 4°C for up to 1 month. Plating of these spore suspensions before and after heat treatment (60°C for 15 min) did not result in significantly different counts. This indicated that the spore suspensions consisted exclusively of spores. The eventual presence of low numbers of vegetative cells did not influence the results of the experiments, since these are more sensitive than spores to pressure and to the other lethal agents used in this work.

Pressure treatment and measurement of germination. Aqueous spore suspensions, diluted fivefold with 50 mM potassium phosphate buffer (pH 7), were pressurized at 40°C in heat-sealed, sterile polyethylene bags. The pressurization equipment (Resato, Roden, The Netherlands) consisted of a manually operated spindle pump, two parallel thermostatted vessels with a circulating water jacket, and the necessary high-pressure valves to allow independent use of both vessels.

To count the ungerminated spores after pressurization, the treated spore suspensions were immersed in water at 60°C for 15 min to inactivate germinated spores. The percentage of ungerminated spores was calculated as the ratio of the number of CFU after germination and heat treatment to the number of CFU before germination but with heat treatment multiplied by 100.

UV treatment. A UV transilluminator (254 nm; 60 W m⁻²) was positioned upside down at approximately 10 cm above an open flat-bottom vial (diameter, 2 cm) containing 1 ml of a spore suspension diluted fivefold in 50 mM potassium phosphate buffer, agitated by a small magnetic stirrer. Samples were withdrawn at different UV exposure times and plated to count survivors.

Measurement of DPA release. The dipicolinic acid (DPA) content of the filtered supernatant of the pressure-germinated spore suspensions was measured by a method based on the characteristic change in the absorbance spectrum of DPA upon addition of Ca²⁺ (21). The total DPA content of the spores was determined after autoclaving the pressure-germinated spore suspensions to completely release DPA (11).

Extraction and immunoblot analysis of SASPs. Spores (for pressure-germinated spores) were suspended in 3 ml of ice-cold 2 N HCl and 6 g of 100-μm-diameter glass beads (Sigma, St. Louis, Mo.) were added. The samples were vortexed five times for 1 min and kept on ice for 3 min between mixing periods. After 10 min on ice, the suspensions were centrifuged for 10 min at 10,000 × g. The pellets were extracted a second time with 3 ml of 2 N HCl for 30 min on ice and finally extracted twice with 2 ml of 3% acetic acid for 20 min at room temperature. The combined HCl and acetic acid extracts were dialyzed in Spectrapor no. 3 tubing (molecular weight cutoff, 3,500) against 1% acetic acid for 62 h at 8°C and lyophilized. The dry residues were dissolved in a small volume of sample buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2.5% sodium dodecyl sulfate, 0.01% bromophenol blue, and 1% dithiothreitol. The samples were run on a sodium dodecyl sulfate-polyacrylamide gel (PhastGel Homogeneous 20; Pharmacia, Uppsala, Sweden). The proteins on the gel were electroblotted onto a polyvinylidene difluoride microporous membrane (Immobilon-P Transfer Membrane; Millipore, Bedford, Mass.). SASPs on the polyvinylidene difluoride membrane were then detected by using rabbit antisera against *B. subtilis* SASP α plus β or *B. subtilis* SASP γ obtained from P. Setlow (University of Connecticut Health Center) and peroxidase-coupled swine anti-rabbit immunoglobulins (Dako a.s., Glostrup, Denmark).

Measurement of ATP. The generation of ATP in pressure-germinated spore suspensions was analyzed by using a firefly luciferase assay (HY-LITE; Merck, Darmstadt, Germany). The ATP content of germinated spores was expressed as the ratio of the number of relative light units after germination to the number of relative light units before germination.

Reproducibility of results. All experiments were done at least in triplicate. The data presented are either means of three replicate experiments or from a single representative experiment.

RESULTS

Germination and inactivation of *B. subtilis* spores by high pressure. To study the pressure dependence of spore germination in *B. subtilis* spores, spore suspensions were subjected to different constant pressures (100 to 600 MPa) at 40°C for 30 min. Germination and inactivation were determined by plating heat-treated and unheated spore suspensions. The fraction of ungerminated spores was almost independent (± 0.5 log unit) of pressure in the pressure range studied (Fig. 1). However, the fraction of survivors showed a minimum at around 200 MPa, while at >400 MPa there was almost no inactivation in spite of considerable germination. This suggested that spores germinated at high pressure would be less sensitive to pressure than those germinated at low pressure. At 100 MPa, we also observed considerable germination but negligible inactivation. However, in this case, the most likely explanation is that a pressure of 100 MPa is too low to have a lethal effect on the germinated spores, since this level of pressure is also generally insufficient to kill vegetative bacteria (4, 20).

In the next set of experiments, we tried to confirm the different pressure sensitivities of spores germinated at low and at high pressures, and we compared the sensitivities of these germinated spores to some other lethal agents.

Sensitivity of spores germinated at low versus high pressure.

(i) **Pressure sensitivity.** The sensitivity of spores germinated at 100 and 500 MPa for 30 min to an increase in pressure to 600 MPa for 10 min was investigated. Pressure was not released to atmospheric pressure before the upshift. Approximately the same level of germination was reached at 100 and 500 MPa, and the upshift to 600 MPa caused little or no additional germination in either case. However, spores germinated at 100 MPa were very sensitive to inactivation at 600 MPa, while spores germinated at 500 MPa remained almost unaffected (Table 1). This result confirmed that while the same level of germination was obtained at low (100 MPa) and high (500 to 600 MPa) pressures, the germinated spores obtained under both conditions differed in sensitivity to pressure killing.

(ii) **Heat sensitivity.** Germination renders spores sensitive to heat. While ungerminated spores survived treatment at 80°C for 15 min (results not shown), pressure-germinated spores were rapidly inactivated at 55°C (Fig. 2A). However, there was no significant difference in the inactivation rate at 55°C for spores germinated at 100 or 600 MPa.

Decimal reduction times could not be calculated because inactivation did not proceed according to first-order kinetics.

(iii) **Hydrogen peroxide sensitivity.** Inactivation of ungerminated spores and spores germinated at 100 and 600 MPa in 0.4 M H₂O₂ was monitored for 10 min (Fig. 2B). The viability of the ungerminated spores was not reduced by this treatment. The 100-MPa-germinated spores were inactivated significantly faster (decimal reduction time $D = 4.1 \pm 0.4$ min) than the 600-MPa-germinated spores ($D = 14.7 \pm 2.1$ min), at least during the first 8 min. After that, a fraction of more resistant

TABLE 1. Sensitivity of *B. subtilis* spores to a pressure of 600 MPa after pregermination at 100 or 500 MPa

Pressure treatment (MPa) (time [min])	Log percentage of:	
	Surviving spores	Ungerminated spores
100 (30)	0.92	-2.16
100 (30) + 600 (10)	-1.83	-2.63
500 (30)	1.47	-1.48
500 (30) + 600 (10)	1.38	-1.19

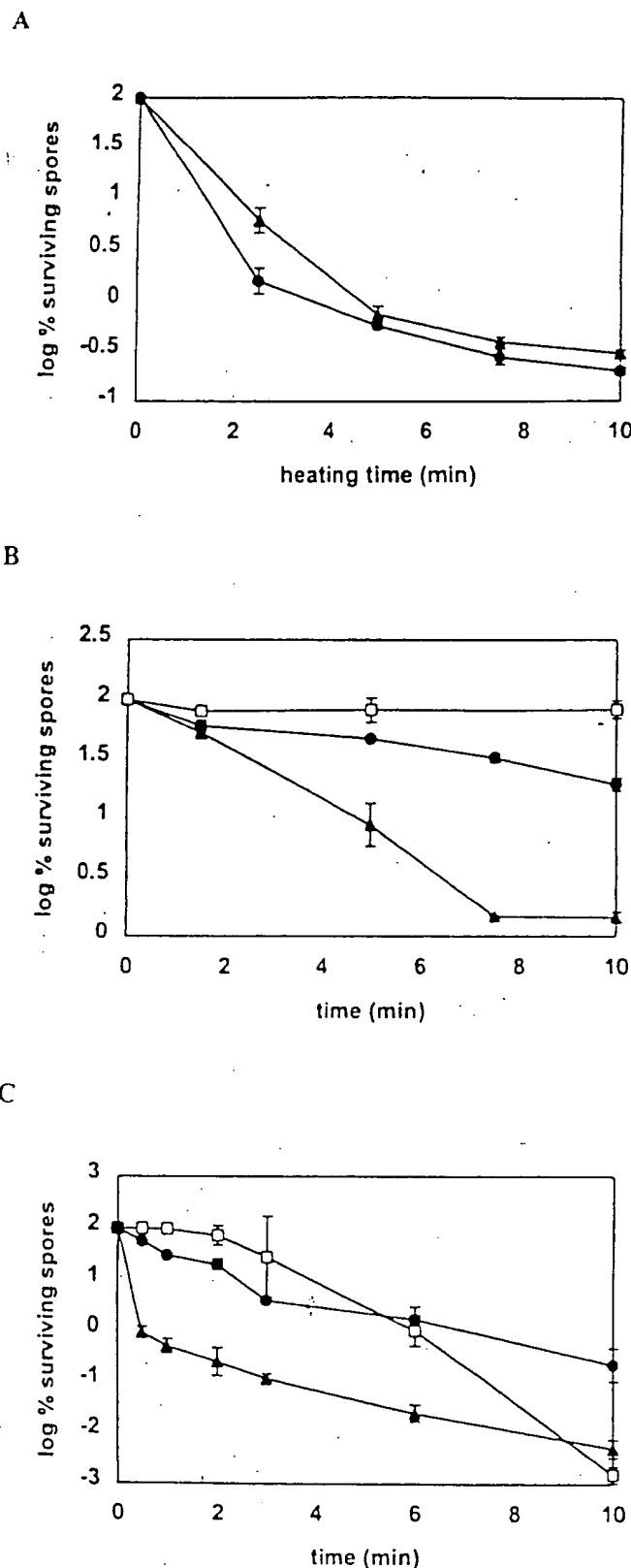


FIG. 2. Inactivation by heat (55°C) (A), hydrogen peroxide (0.4 M) (B), or UV light (C) of ungerminated *B. subtilis* spores (□) and spores germinated at 100 (▲) and 600 (●) MPa.

cells (log % CFU = 0.16) remained in the 100-MPa-treated suspension, that was still 10 times larger than the fraction of ungerminated spores (log % CFU = -0.83). Thus, spores germinated at 100 MPa are more sensitive to H_2O_2 than are those

germinated at 600 MPa, except for a small fraction that remains rather resistant in spite of being germinated.

(iv) **UV sensitivity.** Ungerminated spores were inactivated by UV treatment after a lag phase of 2 min (Fig. 2C). Spores germinated at 600 MPa did not show a lag phase but were more resistant to UV than were the ungerminated spores after their lag phase. The inactivation of spores germinated at 100 MPa occurred at a very high rate during the first 30 s (2-log CFU decrease) and then continued at approximately the same rate as for the 600-MPa-germinated spores. As was the case with the H_2O_2 inactivation, the more UV-resistant fraction of spore populations treated at 100 MPa (log % CFU = -0.03) was larger than the fraction of ungerminated spores (log % CFU = -0.56).

Physiological processes during pressure-induced germination at low versus high pressure. (i) **Release of DPA.** DPA release from the spores was measured after pressure-induced germination at 40°C for 60 min at 100 and 600 MPa. Considerable DPA release occurred with both treatments, but a slightly higher value was noted for the 100-MPa treatment (97.5% of the total spore DPA content) than for the 600-MPa treatment (87.0% of the total spore DPA content). Since the percentage of ungerminated spores was less than 0.1% in both cases, this difference was not due to a difference in germination efficiency.

(ii) **Degradation of SASPs.** The degradation of SASPs during pressure-induced germination was studied by immunoblot analysis of spore suspensions subjected to pressure treatment at 100 or 600 MPa and 40°C for 60 min (Fig. 3).

After pressure treatment at 100 MPa, no α/β -type SASPs were found in the HCl extracts, while a low level was found in the acetic acid extracts. However, after pressure treatment at 600 MPa, α/β -type SASPs were found in the HCl extracts and in the acetic acid extracts. The amount of α/β -type SASPs in the acetic acid extracts was much greater after the 600-MPa treatment than after the 100-MPa treatment.

No γ -type SASPs were found in the HCl extracts or in the acetic acid extracts after pressure treatment at 100 MPa, while after treatment at 600 MPa, γ -type SASPs were found in both extracts.

Since both pressure treatments resulted in a high level of germination (>95%), we can conclude that γ -type SASPs and most of the α/β -type SASPs are degraded in spores germinated at 100 MPa, while SASP degradation is inhibited or delayed in spores germinated at 600 MPa.

A noteworthy observation that was made in these experiments concerns the extraction of the SASPs. As can be seen in Fig. 3, the major amount of SASPs from ungerminated spores was extracted in the first extraction with HCl and only a minor amount was extracted subsequently with acetic acid. For germinated spores in contrast, the acetic acid extract contained the greatest amount of SASPs. Therefore, the SASP contents of spores before and after germination should not be quantitatively compared by these methods.

(iii) **Generation and subsequent consumption of ATP.** Immediately after pressure treatment, i.e., 10 min after pressure release, ATP levels in spore suspensions treated at 100 MPa were considerably increased (35-fold-increased luminescence signal) compared to those of untreated suspensions, whereas no change in ATP levels was found after treatment at 600 MPa. During further storage in potassium phosphate buffer, the ATP content in spores germinated at 100 MPa decreased rapidly during the first hour after pressurization and stabilized at a value about sixfold the value for ungerminated spores. In spores germinated at 600 MPa, the ATP content increased very slowly after pressurization, being about doubled after 200 min.

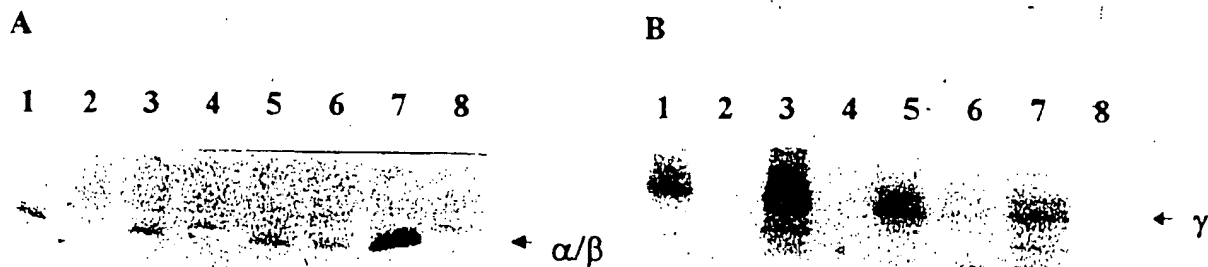


FIG. 3. Immunoblot detection of α/β -type (A) and γ -type (B) SASPs in extracts of *B. subtilis* spores. Lanes: 1 to 4, HCl extracts; 5 to 8, acetic acid extracts. (A) Lanes: 1 and 5, ungerminated PS832 (wild type); 2 and 6, 100-MPa-germinated PS832; 3 and 7, 600-MPa-germinated PS832; 4 and 8, ungerminated PS356 (SASP α/β). (B) Lanes: 1 and 5, ungerminated PS832; 2 and 6, 100-MPa-germinated PS832; 3 and 7, 600-MPa-germinated PS832; 4 and 8, ungerminated PS483 (SASP γ).

The role of SASPs in the pressure resistance of spores. The finding that SASPs are not degraded upon germination at 600 MPa can explain the higher UV and H_2O_2 resistance of 600-MPa-germinated spores compared to spores germinated at 100 MPa (see Discussion) but may also explain the higher pressure resistance of the former. To investigate this hypothesis, *B. subtilis* PS832 (wild type), PS356 (SASP α/β^-), PS483 (SASP γ^-), and PS482 (SASP $\alpha/\beta^- \gamma^-$) spore suspensions were subjected to pressure treatment at 600 MPa and 40°C for 120 min. Germination and inactivation were determined by plating heat-treated and unheated spore suspensions (Table 2). All of the strains showed a high level of germination ($\log \% \text{ CFU} = -3$), while high-pressure inactivation of the SASP α/β^- mutant and the SASP $\alpha/\beta^- \gamma^-$ mutant was significantly ($P < 0.05$) higher than inactivation of the wild type and the SASP γ^- mutant. Thus, α/β -type SASPs seem to provide some protection from high-pressure inactivation of germinated spores.

DISCUSSION

It has been reported by several groups in the 1960's and 1970's that under particular conditions, the inactivation of bacterial spores by high pressure occurs faster at moderate pressure (200 to 300 MPa) than at high pressure (>500 MPa) (5, 7, 19). Since a similar pressure response was also, in many cases (particularly at temperatures below 35°C), observed for pressure-induced germination, a model was proposed in which pressure would cause spores to germinate and lose their pressure resistance, and the germinated spores would subsequently be inactivated by pressure. However, some of the reported data did not support this simple model. For instance, at 45 to 55°C, germination of *B. coagulans* increased with pressure without showing a maximum, whereas inactivation decreased at pressures above 200 to 300 MPa (8, 20). Apparently, high pressure did not inactivate all of the spores it caused to germinate.

In this work, we have observed a similar discrepancy for *B. subtilis*. At 40°C, the degree of pressure-induced germination was roughly constant between 100 and 600 MPa, while

inactivation was maximal at 200 MPa (Fig. 1) and almost non-existent at 600 MPa. Sensitivity of pressure-germinated spores to inactivation by high pressure (600 MPa) was subsequently shown to depend on the pressure used to induce germination. Spores germinated at low pressure (100 MPa) were much more sensitive to pressure inactivation at 600 MPa than were spores germinated at a higher pressure (500 MPa) (Table 1). This can only be explained by postulating a difference in the germination process occurring at 100 versus 500 or 600 MPa, leading to qualitatively different germinated spores.

Nutrient-induced germination has been well described and entails a complex chain of events, leading gradually to more-sensitive spores (Fig. 4) (15, 19, 22, 23, 25). To identify which events occur during pressure-induced germination, the sensitivity of the 100- and 600-MPa-germinated spores to a number of lethal agents was further investigated, as well as the physiological processes occurring during pressure-induced germination at 100 and 600 MPa. For heat sensitivity, no significant differences were observed (Fig. 2A). Since the most important factor believed to determine spore heat resistance is the hydration of the core (25), this result suggests that spores germinated at different pressures have been hydrated to similar degrees.

On the other hand, spores germinated at 100 MPa were much more sensitive to both hydrogen peroxide and UV light than were spores germinated at 600 MPa (Fig. 2B and C). Since the α/β -type SASPs protect spore DNA from various types of damage caused by UV light and hydrogen peroxide (15, 23–25), these results are in agreement with the finding that SASPs are degraded in 100-MPa-germinated spores but not in 600-

TABLE 2. Inactivation and germination of spores of *B. subtilis* SASP mutants after pressure treatment for 120 min at 600 MPa and 40°C

Strain	Log percentage	
	Surviving spores	Ungerminated spores
PS832 (wild type)	0.60 ± 0.3	-2.9 ± 0.3
PS356 (SASP α/β^-)	-0.36 ± 0.3	-3.3 ± 1.0
PS483 (SASP γ^-)	0.55 ± 0.3	-3.1 ± 0.2
PS482 (SASP $\alpha/\beta^- \gamma^-$)	-0.80 ± 0.6	-3.8 ± 0.5

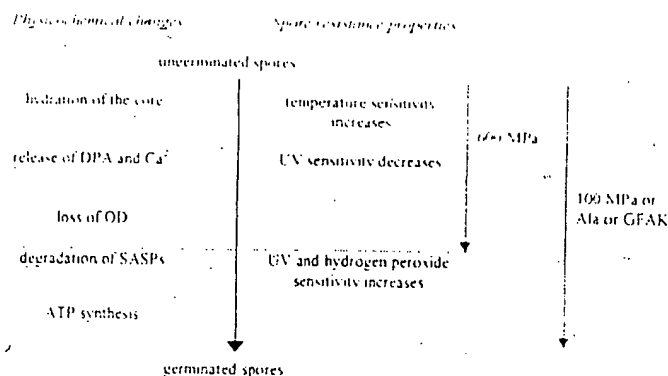


FIG. 4. Scheme of events occurring during nutrient-induced germination (compiled from references 15, 19, 22, 23, and 25 and our own results). As indicated by the dotted lines, our results suggest that 600-MPa treatment causes an incomplete germination process. OD, optical density; GFAK, glucose, fructose, asparagine, and potassium.

MPa-germinated spores (Fig. 3). A possible explanation for the absence of SASP degradation during pressure-induced germination at 600 MPa is that the germination protease that is responsible for SASP degradation (24) may be inactivated at high pressure.

UV resistance of spores is known to be also affected by DPA. DPA was shown to sensitize spore DNA to UV light-induced mutations (22). During nutrient-induced germination, spores lose DPA before SASPs are degraded, which explains why spores in the intermediate germination stage are more resistant to UV light than are dormant spores (24). The release of DPA together with the inhibition of SASP degradation upon pressure-induced germination at 600 MPa then explains why 600-MPa-germinated spores are more resistant to UV light than are ungerminated spores (Fig. 2C).

Finally, the finding that 600-MPa-germinated spores are more sensitive to hydrogen peroxide than are ungerminated spores (Fig. 2B) may be related to the hydration of the core of 600-MPa-germinated spores (18).

An interesting issue is the high-pressure inactivation resistance of the 600-MPa-germinated spores compared to the 100-MPa-germinated spores. One of the prime reasons for the resistance of dormant spores to high-pressure inactivation (at low temperature when no germination takes place) is believed to be the dehydrated state of the core (6, 27). Spore mineralization has also been demonstrated to influence spore heat resistance (3) and pressure-induced germination (2), but its role in pressure resistance remains unclear. Another possibility that was investigated here is that pressure resistance in the 600-MPa-treated spores would be caused by SASPs, which are not degraded under this condition. The results from a comparison of the pressure resistances of spores from various SASP-deficient mutants (Table 2) suggest a slight but significant contribution of α/β -type SASPs to pressure resistance in 600-MPa-germinated spores. γ -type SASPs appear to play no role. Other factors than the α/β -type SASPs are likely to be involved because the mutants lacking α/β -type SASPs were still more resistant to 600-MPa treatment than were wild-type spores pregerminated at 100 MPa.

Whatever the reasons for their high-pressure inactivation resistance, the observation that the 600-MPa-germinated spores are at the same time very heat sensitive but pressure resistant is remarkable in its own right, because vegetative bacterial cells have been generally assumed to be necessarily heat and pressure sensitive (6, 12, 14). Our results reported here, together with results reported earlier for *Escherichia coli* (9), strongly suggest that there is no necessary link between heat and pressure resistance, and this may have important consequences for the use of high pressure as a nonthermal preservation process.

The lack of SASP degradation may indicate that the germination process that is initiated at 600 MPa is arrested at some early stage. Further evidence supporting this view was found by studying the generation of ATP during germination. During the first minutes of nutrient-induced germination, ATP is synthesized from 3-phosphoglycerate that is present in the dormant spore (25). Pressure-induced germination at 100 MPa was also accompanied by rapid ATP generation, while no ATP formation was detected during pressure-induced germination at 600 MPa. Taken together with the results from SASP degradation, this indicates that pressure-induced germination at 100 MPa initiates a number of enzymatic reactions in a similar way as during nutrient-induced germination. During 600-MPa germination, at least two of these enzymatic reactions do not occur, and a possible explanation is that some key enzymes are inactivated at high pressure.

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